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PERFIL METABÓLICO E POTENCIAL ANTIOXIDANTE DE *BRASSICA OLERACEA* VAR. *COSTATA*

Tese de Doutoramento na área de Farmacognosia

Trabalho realizado sob a orientação da Professora Doutora Paula Cristina Branquinho de Andrade

e dos co-orientadores
Professora Doutora Maria de Lourdes Pinho de Almeida Souteiro Bastos e
Professor Doutor Félix Dias de Carvalho

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À memória do meu pai

Trabalho apoiado financeiramente pela Fundação para a Ciência e a Tecnologia, através dos projectos de investigação:

“Caracterização fitoquímica e actividade antioxidante de culturas *in vivo* e *in vitro* de *Brassica oleracea* var. *costata* (couve tronchuda)” (POCTI/AGR/57399/2004)

e

“*Pieris brassicae* como laboratório de síntese de novos compostos com potencial biológico a partir de *Brassica oleracea* var. *costata*, *Brassica oleracea* var. *acephala* e *Brassica rapa* var. *rapa*” (PTDC/AGR-AAM/64150/2006).

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO QUE A TAL SE COMPROMETE.

PUBLICAÇÕES

Fazem parte integrante desta dissertação os seguintes trabalhos já publicados:

*Publicações em **revistas referenciadas** no Journal Citation Reports da ISI Web of Knowledge*:

1. Ferreres, F.; Valentão, P.; Llorach, R.; Pinheiro, C.; Cardoso, L.; Pereira, J. A.; Sousa, C.; Seabra, R. M.; Andrade, P. B., Phenolic Compounds in External Leaves of Tronchuda Cabbage (Brassica oleracea L. var. costata DC). *J. Agric. Food Chem.* **2005,** 53, 2901-2907.

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- Sousa, C.; Valentão, P.; Pereira, J.A.; Ângelo Rodrigues, M.; Bento, A.; Seabra, R.M.; Baptista, P.; Martins, A.; Offermann, J.; Lopes, G.; Pereira, D.M.; Taveira, M.; Andrade, P.B. Caracterização fitoquímica e actividade antioxidante de couve tronchuda, *Brassica oleracea* var. *costata*. Efectuada num dos Seminários 20 Anos de Ensino e Investigação em Ciências Agrárias da Escola superior Agrária do Instituto Politécnico de Bragança. 2 de Maio de **2007**. Bragança (Portugal).

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Em cumprimento do disposto no referido Decreto-Lei, a autora declara que participou activamente na recolha e estudo do material incluído em todos os trabalhos, tendo redigido os textos com a activa colaboração dos outros autores.

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RESUMO

RESUMO

A couve tronchuda (*Brassica oleracea* L. var. *costata* DC) é uma planta da família Cruciferae (ou Brassicaceae), cultivada na Península Ibérica para ser usada em alimentação humana. Entre muitos dos seus atributos relevantes para a saúde, as variedades da espécie *B. oleracea* fornecem nutrientes importantes, como as vitaminas A, C e ácido fólico, os minerais cálcio e potássio e fibras. Adicionalmente, estas couves têm um baixo teor de gorduras e de calorias. Contudo, o conhecimento da composição da couve tronchuda estava limitado aos nutrientes, alguns produtos resultantes do metabolismo primário e aos glucosinolatos.

Nesta dissertação pretendeu-se contribuir para a caracterização do perfil metabólico da couve tronchuda, nomeadamente das folhas internas e externas (divisão realizada de acordo com a variação no fenótipo) e inflorescências, que são o material vegetal usado na alimentação. Para além destas matrizes, pretendeu-se ainda caracterizar as sementes, as plântulas resultantes da sua germinação e os rebentos caulinares obtidos por micropropagação (culturas *in vitro*), sendo que estas duas últimas matrizes podem substituir as couves produzidas em campo para consumo humano. Uma vez que durante o cultivo em campo alguns indivíduos foram atacados por borboletas da espécie *Pieris brassicae* (Lepidoptera: Pieridae), cuja larva se alimenta exclusivamente das folhas de couve e onde os ovos são depositados, avaliou-se o potencial das larvas para sequestrarem e metabolizarem os compostos fenólicos presentes no seu alimento.

O perfil metabólico da couve tronchuda foi caracterizado relativamente às seguintes classes de compostos: polifenóis (abrangendo os ácidos hidroxicinâmicos e os flavonóides), ácidos orgânicos, aminoácidos livres e compostos voláteis, incluindo os isotiocianatos característicos das Cruciferae, os terpenóides e os norisoprenóides, os benzenóides, os fenilpropanóides e os compostos voláteis com origem na degradação dos ácidos gordos. Os metabolitos foram analisados usando técnicas de LC, com detectores de UV-vis, DAD e MS, e por GC, com detector de MS.

Verificou-se que todo o material de *B. oleracea* var. *costata* e *P. brassicae* é muito rico em compostos polifenólicos. O perfil de compostos polifenólicos é característico de cada uma das matrizes estudadas, sendo que as quantidades totais e de cada composto individual variam muito com as condições sob as quais as plantas são obtidas. A principal genina presente nos extractos aquosos de *B. oleracea* var. *costata* e *P. brassicae* é o campferol, com substituintes glicosilados nos hidroxilos dos carbonos 3 e 7 (em alguns casos só em 3). Em alguns dos compostos, a cadeia glicosídica no carbono 3 é acilada com um ou dois ácidos hidroxicinâmicos. Nestes extractos também se identificaram vários heterósidos de ácidos hidroxicinâmicos e ácidos clorogénicos. Verificou-se que no

perfil polifenólico dos extractos das sementes e das plântulas predominam os derivados dos ácidos hidroxicinâmicos; nas folhas internas existem derivados de ácidos hidroxicinâmicos e de flavonóis e as folhas externas, inflorescências e larvas de *P. brassicae* são caracterizadas pela presença de derivados de flavonóis. O perfil polifenólico das amostras obtidas nas culturas *in vitro* (rebentos caulinares) é caracterizado pela presença de vários compostos de cada uma das 3 sub-classes atrás referidas, isto é, derivados de ácidos hidroxicinâmicos, de flavonóis e de ácidos clorogénicos.

Na folha externa de couve tronchuda foram identificados, pela primeira vez na natureza, vários derivados acilados do campferol (campferol 3-*O*-(metoxicafeoil/cafeoil)-soforósido-7-*O*-glucósido, campferol 3-*O*-(sinapoil/cafeoil)-soforósido-7-*O*-glucósido, campferol 3-*O*-(feruloil/cafeoil)-soforósido-7-*O*-glucósido, campferol 3-*O*-(feruloil)-soforotriósido e campferol 3-*O*-(feruloil)-soforósido). Também foi identificado pela primeira vez na natureza um derivado não acilado do campferol, o campferol 3-*O*-tetraglucósido-7-*O*-soforósido. O elevado grau de glicosilação deste último composto é muito invulgar na natureza.

Sabe-se que a composição em metabolitos primários e secundários das plantas é muito influenciada pelas condições em que são obtidas, sendo possível optimizar as condições de cultivo de forma a maximizar a produção de compostos com interesse biológico. Por esta razão avaliou-se a influência de diversos factores de produção no perfil metabólico de algumas das matrizes atrás referidas. Para as folhas internas e externas avaliou-se a influência de parâmetros agronómicos, como produção orgânica ou convencional, adição de fertilizantes químicos e época de colheita, no perfil de compostos fenólicos, ácidos orgânicos, aminoácidos livres e compostos voláteis. De uma forma geral, as amostras de *B. oleracea* var. *costata* produzidas sem adição de fertilizantes ou com fertilização orgânica contêm maiores concentrações de metabolitos secundários. Para os rebentos caulinares, o principal objectivo foi optimizar a produção de compostos polifenólicos. Das várias condições de cultura testadas o meio líquido basal “Murashige and Skoog” (MSM), suplementado com 2 mg/L de benziladenina (BAP) e 0,1 mg/L de ácido 1-naftalenoacético (NAA) permitiu obter rebentos caulinares com a maior variedade de metabolitos polifenólicos.

A grande variedade de compostos presentes nos extractos de várias matrizes (no total foram identificados 79 compostos polifenólicos) e as concentrações atingidas (nos extractos aquosos de folhas externas de plantas produzidas em regime de agricultura biológica os compostos polifenólicos atingiram 30 g/kg de peso de extracto seco), permitem concluir que, na dieta tradicional Portuguesa, a couve tronchuda contribui

significativamente para a ingestão de compostos fenólicos e para os efeitos benéficos associados a estes compostos.

As larvas de *P. brassicae* foram também caracterizadas relativamente à composição fenólica, tendo-se verificado que são capazes de metabolizar e sequestrar os compostos fenólicos que obtêm da couve tronchuda, exibindo um perfil de compostos fenólicos diferente do da planta hospedeira.

Relativamente ao perfil de ácidos orgânicos observou-se uma menor variabilidade de compostos quando comparado com o dos compostos fenólicos. De uma forma geral o perfil metabólico é caracterizado pela presença de 2 ácidos orgânicos predominantes, os ácidos cítrico e málico, que representam mais de 90% dos ácidos orgânicos identificados. Outros compostos comuns a todas as matrizes, embora minoritários, são os ácidos aconítico, xiquímico e fumárico, estando presentes em quantidades da ordem dos mg/kg de extracto liofilizado.

O perfil de aminoácidos livres da couve tronchuda é muito característico. Na folha externa os aminoácidos maioritários são a prolina e a arginina. Nas folhas internas os aminoácidos maioritários são a arginina e a treonina. Os aminoácidos usualmente maioritários nas plantas, ácidos aspártico e glutámico, asparagina e glutamina, representam, cada um, menos de 10% dos aminoácidos livres em todos os extractos analisados. A concentração de aminoácidos livres nos extractos de couve tronchuda atinge 14,4 g/kg de peso fresco, uma concentração muito superior à que habitualmente se encontra nas plantas (20-200 mg/kg de peso fresco).

O perfil qualitativo e quantitativo de aminoácidos livres variou de acordo com o grau de desenvolvimento da folha: as quantidades de valina, prolina, arginina, leucina, cisteína, lisina, histidina e tirosina são significativamente diferentes nos extractos aquosos de folha interna e de folha externa.

Os compostos voláteis e semi-voláteis identificados nos extractos liofilizados de couve tronchuda pertencem a muitos grupos diferentes, incluindo acetais, ácidos gordos, aldeídos, alcoóis, ésteres metílicos e etílicos, cetonas, isotiocianatos, tiocianatos, sulfuretos, terpenóides e norisoprenóides, nitrilos, benzenóides e fenilpropanóides. Ao todo, foram identificados 71 compostos, entre os quais 16 compostos de enxofre com origem provável na degradação dos glucosinolatos.

Entre os compostos de enxofre foram identificados os isotiocianatos de alilo e de butenilo, provenientes da hidrólise dos glucosinolatos alifáticos sinigrina e gluconapina, previamente descritos na couve tronchuda.

De um modo geral, a quantidade de compostos de enxofre nas folhas internas é superior à das folhas externas. A fertilização com enxofre aumenta a quantidade de compostos de enxofre na fracção volátil dos extractos aquosos de couve tronchuda.

O segundo grande objectivo desta dissertação foi a avaliação do potencial antioxidante da couve tronchuda. Para isso, aferiu-se a capacidade de captação de várias espécies reactivas, nomeadamente os radicais DPPH, superóxido, hidroxilo e óxido nítrico, ácido hipocloroso e peroxinitrito das matrizes previamente caracterizadas. Também se avaliou a capacidade de algumas destas matrizes inibirem a xantina oxidase.

De uma forma geral os extractos demonstraram ter uma actividade dependente da concentração, numa gama relativamente larga de concentrações. A couve tronchuda demonstrou capacidade para interceptar estas espécies, embora a actividade antioxidante contra o ácido hipocloroso tenha sido pouco significativa.

As larvas de *P. brassicae* demonstraram ter uma capacidade considerável para inibir a xantina oxidase, ao contrário da *B. oleracea* var. *costata* de que se alimentam. Genericamente, a actividade antioxidante da larva de *P. brassicae* foi melhor do que a da couve que lhe serviu de alimento, e este resultado deu origem a uma patente para a obtenção de antioxidantes naturais com possível aplicação na indústria cosmética, alimentar e dos plásticos.

Os resultados do potencial antioxidante dos extractos de couve tronchuda observados quimicamente (folha externa de *B. oleracea* var. *costata* produzida num sistema de agricultura biológica) observados quimicamente não foram confirmados num ensaio realizado com hepatócitos primários de rato, submetidos a stress oxidativo. Quando se avaliou o efeito do extracto hidrolisado nos hepatócitos expostos ao paraquato (10 mM), verificou-se que embora em quantidades mais baixas (200 µg/mL) o extracto tivesse uma acção protectora ligeira, para concentrações mais elevadas os efeitos avaliados pelos indicadores de viabilidade celular (MTT, LDH) e de stress oxidativo, nomeadamente os níveis de glutationa e de ATP, foram agravados. Estes resultados podem ser explicados pela insuficiência de NADH e NADPH nas células para destoxificar os compostos quinónicos formados pela oxidação do campferol, uma vez que o NADH e o NADPH são consumidos no ciclo redox do paraquato pelas diaforases celulares. A inibição pelo extracto da activação do NFkB, induzida pelo stress oxidativo, pode também justificar os resultados obtidos, uma vez que este factor de transcrição está envolvido na regulação de genes envolvidos na defesa celular.

Adicionalmente, o extracto, por si só, não é tóxico para os hepatócitos em nenhuma das concentrações testadas e induz a QR.

Em conclusão, no perfil metabólico da couve tronchuda foram identificados muitos compostos para os quais estão descritas actividades biológicas, nomeadamente antioxidante, anti-inflamatória e anti-microbiana. A actividade antioxidante de extractos de couve tronchuda foi confirmada em ensaios químicos realizados com diferentes espécies

reactivas. Porém, num sistema celular, verificou-se que os extractos podem potenciar a toxicidade de agentes pró-oxidantes como o paraquato. Estes resultados demonstram que as propriedades antioxidantes observadas em sistemas não celulares, nem sempre se confirmam em sistemas celulares, em que as concentrações de extracto necessárias para neutralizar as espécies pró-oxidantes podem causar efeitos nefastos às células.

ABSTRACT

ABSTRACT

Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) belongs to Cruciferae (or Brassicaceae) family, being cultivated in the Iberian Peninsula for human nutrition. Tronchuda cabbage is considered a healthy vegetable, constituting an important source of the vitamins A, C and folic acid, calcium, potassium, and fibre. Another relevant feature of tronchuda cabbage is its low content in fat and energy. However, the knowledge of tronchuda cabbage composition was restricted to nutrients, some primary metabolites and glucosinolates.

With this thesis we intend to contribute for the characterization of the metabolic profile of tronchuda cabbage, namely its edible parts, internal and external leaves (division made according to the phenotype) and inflorescences. In addition, seeds, sprouts and shoots were also characterized, bearing in mind that these last two can replace tronchuda cabbage consumption. Since some individuals in the field were infested with the larvae of *Pieris brassicae* L. (Lepidoptera: Pieridae), the ability of the larvae to scavenge and metabolise the phenolic compounds present in its feedstuff was also evaluated.

The metabolic profile of tronchuda cabbage extracts was characterized in what concerns to the following classes of compounds: polyphenolics (comprising hydroxycinnamic acids and flavonoids), organic acids, free amino acids and also volatile and semi-volatile compounds, including isothiocyanates, terpenoids, norisoprenoids, benzenoids, phenylpropanoids, and compounds derived from fatty acid degradation. LC-UV, LC-DAD, LC-MS and GC-MS techniques were applied in the analysis of the metabolites present in *B. oleracea* var. *costata* and *P. brassicae* extracts.

It was verified that *B. oleracea* var. *costata* and *P. brassicae* material are very rich in polyphenolic compounds. Each material exhibited a distinct polyphenolic profile and total and individual amounts of each compound varied with plant culture conditions. The polyphenolic profile of *B. oleracea* var. *costata* and *P. brassicae* aqueous extracts was characterized by the presence of kaempferol derivatives, with substituents on the hydroxyl on carbon 3 or 3 and 7. The substituent groups contained a variable number of sugar units, and the sugar moiety on the hydroxyl on carbon 3 can be acylated with one or two hydroxycinnamic acids. Non-flavonoidic heterosides of hydroxycinnamic acids and chlorogenic acids were also identified. In general, in the phenolic profile of seeds and sprouts extracts hydroxycinnamic acids are prevalent, in internal leaves extracts both hydroxycinnamic acids and flavonol derivatives are present, while the phenolic profile of external leaves, inflorescences and larvae extracts is characterized by the presence of flavonol derivatives. The phenolic profile of shoots is characterized by the presence of

several compounds of each sub-class (hydroxycinnamic acids, flavonol derivatives and chlorogenic acids).

Several of the kaempferol acylated derivatives present in the external leaves aqueous extracts were identified for the first time in nature, namely kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl)-sophorotrioside and kaempferol 3-*O*-(feruloyl)-sophoroside. Kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside, which has an unusual high degree of glycosylation, was also identified for the first time in nature.

It is well known that primary and secondary metabolites composition in plants depend upon the culture conditions, which can be optimized in order to maximize the production of specific compounds with biological interest. For this reason the influence of several production factors, like fertilization regimen and harvesting season, on the metabolic profile (phenolic compounds, organic acids, free amino acids and volatiles) of tronchuda cabbage internal and external leaves was evaluated. In general, *B. oleracea* var. *costata* produced with no fertilization or with biological fertilization exhibited higher concentrations of secondary metabolites. In order to optimize the phenolic compounds production by shoots several *in vitro* culture conditions were tested. Best results were obtained with liquid “Murashige and Skoog” basal medium (MSM), supplemented with 2 mg/L of benzylaminopurine (BAP) and 0.1 mg/L of 1-naphthaleneacetic acid (NAA).

The huge variety of compounds in the analyzed extracts of the several matrices (overall 79 different phenolic compounds were identified) and the attained concentrations (30 g/kg dry matter extract in external leaves produced under a biological regimen) allow to conclude that, in the traditional Portuguese diet, tronchuda cabbage can be a major source of phenolic compounds, contributing for the beneficial effects associated to these compounds.

P. brassicae larvae extract’s characterization showed that larvae are able to sequester and metabolize the phenolic compounds obtained from tronchuda cabbage, exhibiting a phenolic profile different from that of its host plant.

The organic acids profile of tronchuda cabbage extracts showed lower variability than that of the phenolic compounds. In general, this profile is characterized by the presence of 2 prevailing acids, citric and malic, which accounted for more than 90% of the identified acids. Aconitic, chiquimic and fumaric acids, although present in lower amounts (in the range of mg/kg of dried extract), were common to all assayed samples.

The free amino acids profile of tronchuda cabbage is very characteristic. In external leaves proline and arginine are predominant, while in the internal leaves arginine and threonine are the main compounds. The major amino acids usually found in other

plants, aspartic and glutamic acids and asparagine and glutamine, represent less than 10% of free amino acids in tronchuda cabbage extracts. Free amino-acids concentration reached 14.4 g/kg fresh weight, which is higher than the concentration commonly exhibited by other plants (20-200 mg/kg fresh weight).

Free amino-acids profile varied according to the development stage of the leaves: the contents of valine, proline, arginine, leucine, cysteine, lysine, histidine, and tyrosine of internal and external leaves aqueous extracts were significantly different.

The volatiles and semi-volatiles identified in tronchuda cabbage lyophilized extracts belong to different groups, including acetals, fatty acids, aldehydes, alcohols methyl and ethyl esters, ketones, isothiocyanates, thiocyanates, sulfides, terpenes, norisoprenoids, nitriles, benzenoids and phenylpropanoids. Overall, 71 compounds were identified, including 16 sulphur compounds with probable origin in the degradation of glucosinolates. The allyl and butyl isothiocyanates, originated from the hydrolysis of the aliphatic glucosinolates sinigrin and gluconapin (previously identified in tronchuda cabbage), were present in the extracts' volatile fraction.

In a general way, the amount of sulphur compounds in internal leaves is superior to that of the external ones. Sulphur fertilization increases the content of sulphur compounds in the volatile fraction of the aqueous extracts.

The second major purpose of this thesis was the evaluation of the antioxidant potential of tronchuda cabbage. For this reason the scavenging capacity of previously characterized extracts towards several reactive species, including DPPH, superoxide, hydroxyl and nitric oxide radicals, hypochlorous acid and peroxynitrite, was evaluated. Xanthine oxidase inhibition was also assessed. In general, the extracts exhibited a concentration dependent scavenging capacity, in a wide concentration range. The extracts proved to scavenge the assayed reactive species, although activity against hypochlorous acid was not relevant.

P. brassicae larvae extracts were able to inhibit xanthine oxidase, which was not observed for the host plant extract. Generically, the antioxidant potential of *P. brassicae* larvae extracts was higher than that of tronchuda cabbage. This result justified a patent for the production of natural antioxidants with applicability in cosmetic, food and plastic industries.

The antioxidant properties observed in non-cellular assays of tronchuda cabbage extracts (external leaves of *B. oleracea* var. *costata* obtained under organic production) were not confirmed in a biological assay, made with primary rat hepatocytes subjected to oxidative stress, induced by paraquat (10 mM). Although some degree of protection was noticed with extract concentrations of 200 µg/mL, an obvious potentiation of PQ-induced

toxicity at higher extract concentrations was verified by compromised cell viability (assessed with MTT and LDH assays) and GSH and ATP indexes. These results can be explained by the cellular lack of NADH and NADPH needed to destoxify the quinonic compounds resulting from kaempferol oxidation, considering that the exposure of hepatocytes to PQ leads to depletion of NADH and NADPH, necessary for the redox cycle of paraquat by cellular diaphorases. Also the inhibition by the extract of NF-κB activation induced by paraquat may explain these results, since NF-κB is involved in the regulation of immune and defence genes necessary to overcome cellular insults.

Cellular exposure to the extract alone proved to be not toxic to the hepatocytes in the concentration range tested. Additionaly, it was able to induce QR.

In conclusion, in the metabolic profile of tronchuda cabbage, several compounds with known biological activity, like antioxidant, antimicrobial and anti-inflammatory, were identified. Antioxidant activity of the extracts was confirmed in cell-free assays with different reactive species. However, it was observed that the extracts can actually potentiate the toxicity of pro-oxidant agents like paraquat in a cellular system. These results highlight that the antioxidant effects observed in cell free systems are not always confirmed in cellular systems, in which the concentrations required to scavenge pro-oxidant species may be highly detrimental to the cells.

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ABREVIATURAS E SÍMBOLOS

ABREVIATURAS E SÍMBOLOS

$\lambda_{máx}$	Comprimento de onda de absorção máxima
[M-H]	Ião associado ao peso molecular
$[M-H]^-$	Ião pseudomolecular
2,4D	Ácido 2,4 diclorofenoxiacético
4CL	4-Cumarato:coenzima A liase
ABTS	2,2'-azinobis-(3-etilbenzotiazoline-6-sulfonato)
ACCase	Acetil CoA carboxilase
ADME	Absorção, distribuição, metabolismo e excreção
ANS	Antocianidina sintetase
APCI	Ionização química à pressão atmosférica (de a*tmospheric pressure chemical ionization*)
API	Ionização à pressão atmosférica (de a*tmospheric pressure ionization*)
APPH	2,2'-azobis (2-amidinopropano)
APPI	Foto-ionização à pressão atmosférica (de a*tmospheric pressure photoionisation*)
ARE	Elemento de resposta antioxidante (de a*ntioxidant response element*)
EpRE	Elemento de resposta a compostos electrofílicos (de e*lectrophile response element*)
AS	Aureusidina sintetase
ATP	Adenosina trifosfato (de a*denosine triphosphate*)
BAP	Benziladenina (de *benzylaminopurine*)
B5	Meio de cultura B5 de Gamborg (de *Gamborg's B5 media*)
C3H	*p*-Cumarato 3-hidroxilase
CA4H	Cinamato 4-hidroxilase
CAM	Metabolismo crassuleano (de c*rassulacean acid metabolism*)
CAT	Catalase
CCD	Dioxigenases de quebra de carotenóides (de *carotenoid cleavage dioxygenases*)
CCOMT	Cafeoil *O*-metiltransferase
CE	Electroforese capilar (de *capillary electrophoresis*)
CGA	Ácidos clorogénicos (de *chlorogenic acids*)
CHI	Chalcona isomerase
CHS	Chalcona sintetase
CI	Ionização química (de *chemical ionization*)
CID	Decomposição induzida por colisão (de *collision induced decomposition*)

Cis	Cisteína
CoA	Coenzima A
COMT	Catecol *O*-Metil-Transferase
CYP	Enzimas de biotransformação do citocromo P450 (de *cytochrome* P450)
DAD	Detector de díodos (de *Diode Array Detector*)
DAHP	3-desoxi-arabinoheptulsonato-7-fosfato
DFR	di-hidroflavonol 4-redutase
DIMS	Espectrometria de massa com injecção directa (de *Direct Injection Mass Spectrometry*)
DMADP	Dimetilalil difosfato (de *dimethylallyl diphosphate*)
DMID	7,2'-dihidroxi, 4'-metoxiisoflavanol desidratase
DMSO	Dimetilsulfóxido
DOXP	1-desoxi-D-xilulose fosfato (de *1-deoxy-D-xylulose 5-phosphate*)
DPPH	1,1'-difenil-2-picrilhidrazilo (de *1,1'-diphenyl-2-picrylhydrazyl*)
EGF	Factor de crescimento epidérmico (de *Epidermal Growth Factor*)
EI	Impacto electrónico (de *Electron Impact*)
EPSP	sintetase de 5-enolpiruvoilxiquimato 3-fosfato (*de 5-enolpyruvylshikimate-3-phosphate synthase*)
ESI	Ionização por electrões em vapor (de *electrospray ionization*)
F3'5'H	Flavonóide 3'5'-hidroxilase
F3'H	Flavonóide 3'-hidroxilase
F3H	Flavanona 3-hidroxilase
F5H	Feruloil 5-hidroxilase
FDP	Difosfato de farnesilo (de farnesyl diphosphate)
Fen	Fenilalanina
FLS	Flavonol sintetase
FS1/FS2	Flavona sintetase
FT-ICR	Transformada de Fourrier – Ressonância iónica de ciclotrões (de *Fourier transform ion cyclotron resonance mass spectrometry*)
GA	Giberelinas (de *gibberellic acid*)
GC	Cromatografia gasosa (de *gas chromatography*)
GDP	Difosfato de geranilo de (*geranyl diphosphate)*
GGDP	Difosfato de geranilgeranilo (de *geranylgeranyl diphosphate*)
Glu	Glucose
GPx	Glutationa Peroxidase
GSH	Glutationa
GSSG	Glutationa Oxidada

GST	Transferases da Glutationa (de *glutathione transferases*)
GT	Glicosiltransferases
HCA	Ácidos hidroxicinâmicos (de *hydroxycinnamic acids*)
HCT	Hidroxicinamoiltransferase
Hsp	Proteínas de choque térmico (de *heat shock protein*)
I/R	Isquémia / Reperfusão
I2'H	Isoflavona 2'-hidroxilase
IAA	Ácido indolacético (de *indoleacetic acid*)
IC_{50}	Concentração Inibitória de 50% da reacção
IFR	Isoflavona redutase
IFS	Isoflavona sintetase
IGF	Factores de crescimento semelhantes à insulina (de *insulin-like growth factor*)
IL	Interleucina
IOMT	Isoflavona *O*-metiltransferase
IPP	Difosfato de isopentenilo (de *isopentyl diphosphate*)
Iso	Isoleucina
JNK	Cinase c-Jun (de *c-Jun kinase*)
LAR	Leucoantocianidina redutase
LC	Cromatografia líquida (de *liquid cromatography*)
LDH	Lactato desidrogenase
LDOX	Leucoantocianidina dioxigenase
LETF	Factores de transcrição predominantes no fígado (de *liver-enriched transcription factors*)
Leu	Leucina
m/z	Relação massa/carga dos fragmentos iónicos formados
MAPK	Cinases activadas por compostos mitogénicos (de *mitogen-ativated protein cinase*)
Met	Metionina
MRP	Proteínas associadas à multiresistência a xenobióticos (de *multidrug resistance associated protein*)
MS	Espectrometria de massa, espectro de massa (de *mass spectrometry*)
MS^2	Espectro de massa da fragmentação do ião molecular desprotonado
MS^3	Espectro de massa da quebra dos fragmentos mais significativos derivados de $[M-H]^-$
MSM	Meio de cultura de Murashige and Skoog (de *Murashige and Skoog basal medium*)

MTT	Brometo de 3-[4,5-dimetiltiazol-2-il]-2,5-difeniltetrazólio (de *3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*)
MVA	Via acetato / mevalonato
NAA	Ácido 1-naftalenoacético (de *1-naphthaleneacetic acid)*
NADH	Nicotinamida Adenina Dinucleotídeo (forma reduzida)
nd	Não detectado
NF-kB	Factor nuclear kB (de *nuclear factor kB*)
NMR	Ressonância magnética nuclear (de *nuclear magnetic resonance*)
NO	Óxido nítrico (de *nitric oxide*)
NOS	Oxido nítrico sintetase (de *Nitric oxide synthase*)
nq	Não quantificado
OMT	*O*-metiltransferase
PAL	Fenilalanina amónia liase (de *phenylalanine ammonia lyase*)
PCA	Análise de Componentes Principais (de *principal component analysis*)
PDGF	Factor de crescimento derivado das plaquetas (de *platelet-derived growth factor*)
PEP	Fosfoenolpiruvato (de *phosphoenolpyruvate*)
P-gp	Glicoproteína-P
PLS-DA	análise discriminatória parcial dos mínimos quadrados (de *partial least squares discriminatory analysis*)
PMS	Metassulfato de fenazina (de *phenazine methosulfate*)
PPO	Polifenol oxidases
PQ	Paraquato
QR	Quinona redutase
R	Coeficiente de correlação
RamT	Ramnosilo transferase
RNS	Espécies reactivas de azoto (de *reactive nitrogen species*)
RT	Tempo de retenção (de *retention time*)
SAPK	Cinase activada pelo stress (de *stress-activated protein kinase*)
SCE	Sinapoilcolina esterase
SCT	1-*O*-sinapoilglucose:colina sinapoiltransferase
SGLT1	Transportador de glucose dependente do sódio
SGT	Sinapato glusosiltransferase
SMT	1-*O*-sinapoilmalato:colina sinapoiltransferase
SNP	Nitroprussiato de sódio (de *sodium nitroprussiate*)
SOD	Superóxido dismutase
TGF	Factor de transformação de crescimento (de *transforming growth factor*)

Tir	Tirosina
TNFα	Factor tumoral de necrose α (de *tumor necrosis factor α*)
TOF	Tempo de voo (de *time-of-flight*)
TPTZ	2,4,6-tripiridil-*S*-triazina
Tre	Treonina
Tri	Triptofano
UFGT, UDP	Flavonóide glucosilo transferase
UGT	UDP-glucuronosiltransferase
UV	Ultra-violeta
Val	Valina
Vis	Visível
VR	Vestitona redutase
X	Xantina
XO	Xantina Oxidase
XRE	Elemento de resposta aos xenobióticos (de *xenobiotic response element*)

PARTE I

ESTRUTURA GERAL DA TESE

INTRODUÇÃO

OBJECTIVOS

1. ESTRUTURA GERAL DA TESE

A presente dissertação encontra-se dividida em 4 partes principais

PARTE I – Introdução geral e objectivos da dissertação

Neste capítulo faz-se uma introdução aos temas que foram objecto de estudo desta dissertação. O desenvolvimento de cada tema é feito de uma forma geral, sendo aprofundados os aspectos mais importantes para a interpretação dos resultados obtidos que se encontram na secção experimental. No final do capítulo são enumerados os principais objectivos da dissertação.

PARTE II- Secção experimental

Esta secção encontra-se dividida em 17 capítulos, correspondendo aos artigos, publicados ou submetidos, no âmbito desta dissertação.

PARTE III – Discussão geral e conclusões

Esta secção integra os resultados dos diferentes trabalhos e tenta relacioná-los com os trabalhos existentes que abordam assuntos semelhantes.

As conclusões a que os diversos trabalhos realizados permitiram chegar encontram-se sumariadas neste capítulo.

PARTE IV – Referências bibliográficas

As referências necessárias à elaboração desta dissertação encontram-se nesta última secção.

2. INTRODUÇÃO

2.1. Introdução geral

O metaboloma das plantas, resultado final do genoma, apresenta uma complexa variabilidade dinâmica de compostos primários e secundários (Figura 1). Apesar de existirem plataformas tecnológicas para estudar os genomas, transcriptomas e até os proteossomas, a obtenção de metabolomas genuínos é difícil, devido à grande diversidade química dos metabolitos. Contudo, o metaboloma apresenta vantagens específicas no estudo dos fenótipos de sistemas biológicos, nomeadamente na análise de sistemas dinâmicos por ser mais fácil extrair relações causa-efeito (1).

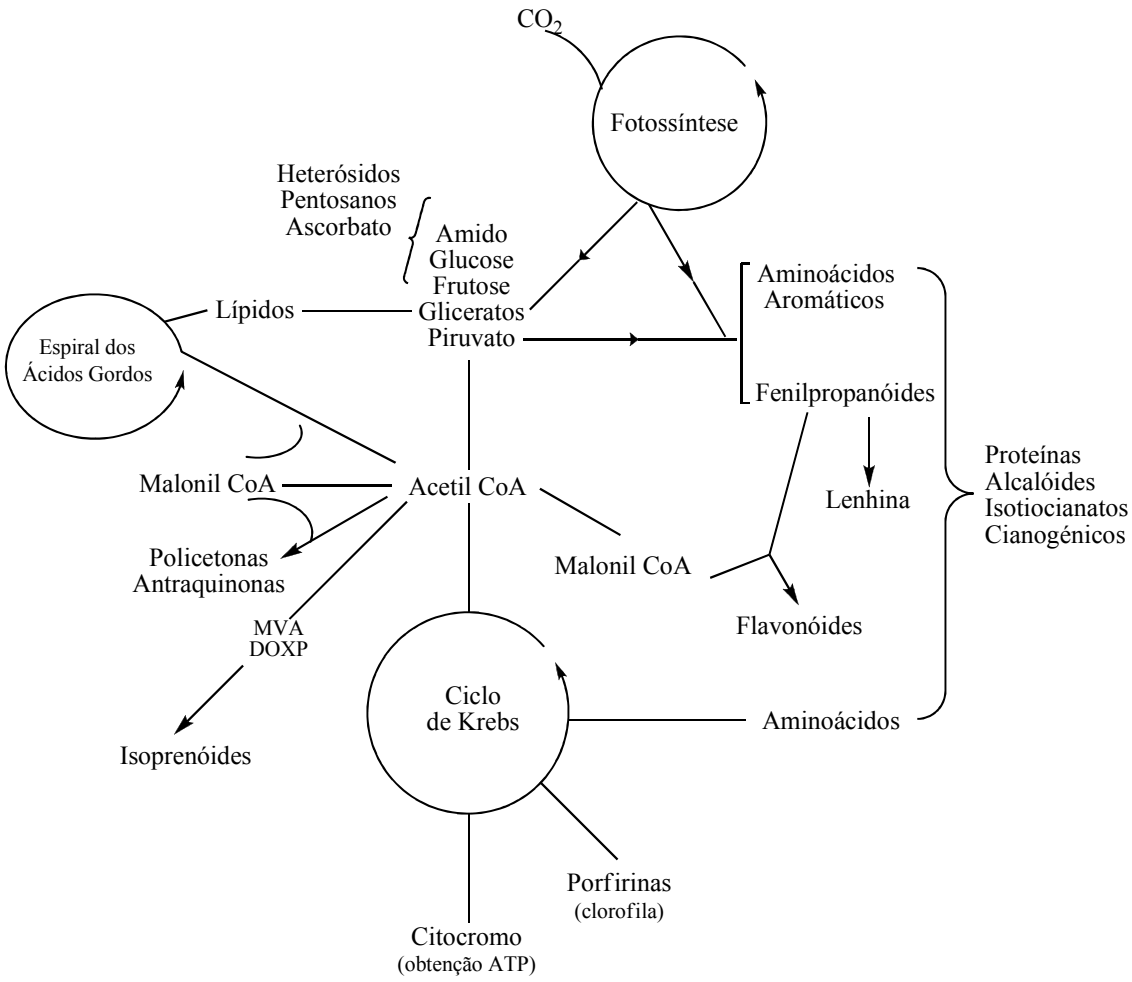


Figura 1. Origem de alguns metabolitos secundários relativamente às vias metabólicas básicas, [adaptada de (2)].

A espectrometria de massa é uma plataforma analítica com um papel muito importante na evolução da metabolómica e, quando combinada com sistemas cromatográficos de elevada resolução, permite a multi-detecção de analitos, com elevada sensibilidade e especificidade. O tamanho e complexidade dos conjuntos de dados obtidos tornam necessários métodos quimiométricos e bioinformáticos que permitam obter variações sistemáticas (3-5).

Em geral, a cromatografia gasosa com detector de massa (GC-MS) é eficaz na análise de compostos hidrofóbicos de peso molecular relativamente baixo, tais como os hidrocarbonetos e ésteres de metabolitos com polaridade reduzida. A eficácia desta técnica é parcial em relação aos metabolitos de elevado peso molecular, mas tem a vantagem de permitir a medição directa dos compostos voláteis, usando técnicas de "head-space" (3). A cromatografia líquida com detector de massa (LC-MS) é útil na análise de metabolitos polares com peso molecular baixo a moderado (<2000) incluindo iões metálicos e alcalóides, esteróides, ácidos gordos, ácidos orgânicos, aminoácidos e compostos polifenólicos. Em particular, o LC-MS equipado com "electrospray ionization", ESI-LC-MS, é uma técnica de excelência na análise de heterósidos flavonóidicos.

A análise metabolómica pode virtualmente ser feita em todos os tecidos, desde células em cultura a plantas intactas, incluindo o meio circundante (3).

O fluxograma de obtenção do metaboloma envolve o processo de colheita e preparação da amostra, recolha de dados analíticos, pré-processamento dos dados em bruto e a análise e armazenamento de dados (Figura 2) (5).

A revolução das ciências "ómicas" permite uma investigação sistemática de misturas complexas e permite especificamente ligar as análises fitoquímicas com outras estratégias (como a triagem *in vitro* ou *in vivo* da actividade biológica, toxicidade, diversidade morfológica das plantas e parâmetros ecológicos) (6).

A metabolómica pode ter uma grande variedade de aplicações, tais como a incrementação dos fluxos metabólicos em vias bioquímicas seleccionadas pela engenharia metabólica, para aumentar o valor nutricional dos alimentos ou para a produção de produtos farmacêuticos (7).

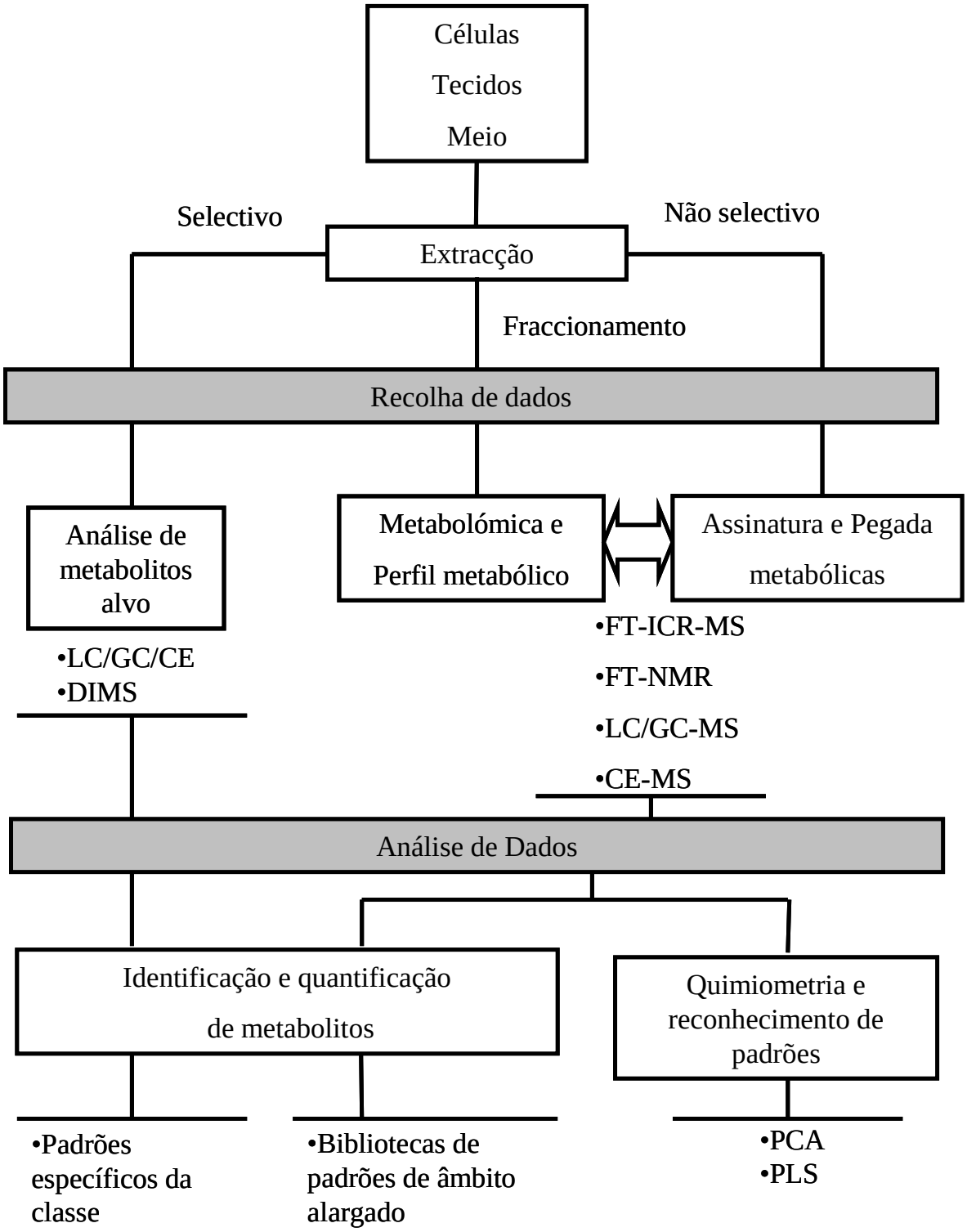


Figura 2. Fluxograma das várias metodologias usadas na análise metabolómica [adaptada de (3)].

2.1.1. Descrição da couve tronchuda

A couve tronchuda (*Brassica oleracea* L. var. *costata* DC) é uma planta da família *Cruciferae* (ou Brassicaceae) do género *Brassica*, cultivada na Península Ibérica para ser usada na alimentação humana.

As crucíferas são herbáceas ou sub-arbustos compreendendo cerca de 350 géneros e 3000 espécies, algumas delas cultivadas desde a pré-história.

A família Cruciferae inclui muitos vegetais comuns, como os brócolos, couve-flor, couve, nabiça, couve de Bruxelas, rabanete e várias mustardas (8). O consumo de crucíferas é relativamente elevado, por comparação com outros vegetais, e variável dependendo da região geográfica.

Entre as culturas comercialmente importantes existem muitas variedades pertencentes a seis espécies do género *Brassica*, sendo as relações evolutivas dos seus genomas descritas pelo triângulo de U (Figura 3), (9). A teoria do triângulo de U baseia-se na ideia de que a combinação de três genomas ancestrais diferentes criaram muitos dos vegetais e oleaginosas dos nossos dias. As três espécies diplóides, consideradas primitivas ou básicas (*Brassica rapa*, *Brassica nigra* e *Brassica oleracea*), representadas pelos genomas AA, BB e CC cruzaram-se, originando os híbridos tetraplóides (*Brassica carinata*, *Brassica juncea* e *Brassica napus*) (10).

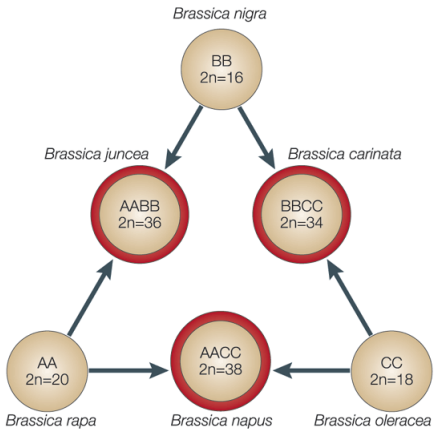


Figura 3. Relação evolutiva dos genomas de espécies do género *Brassica* [Triângulo de U, retirada de (9)]. 2n. Número de cromossomas diplóides.

Algumas das principais espécies de *Brassica* diplóides são:

B. rapa (AA - n=10)**:** couve chinesa, nabo.

B. nigra (BB - n= 8): mostarda preta.

B. oleracea (CC - n= 9): couve, brócolos, couve-flor.

As espécies de *Brassica* tetraplóides (anfidiploídes das três espécies anteriores) comercialmente mais importantes são:

B. juncea (AABB - n=18): mostarda da Índia.

B. napus (AACC - n=19): couve-nabiça, colza.

B. carinata (BBCC - n=17): mostarda da Etiópia.

As espécies em estado selvagem evoluíram para uma gama de variedades em que diferentes partes da planta se tornaram partes edíveis (11). Algumas variedades de couves são usadas sobretudo em sopas e como acompanhamento de diversos pratos. Outras possuem um aroma forte e picante sendo usadas como condimento (mostarda) ou em saladas [rúcula (*Eruca sativa*)]. Existe ainda a colza que é uma conhecida oleaginosa.

A couve tronchuda (*B. oleracea* L. var. *costata* DC) é caracterizada por caules curtos e grossos, com folhas e nervuras largas. Em termos agrícolas, esta couve dá produções elevadas, é pouco susceptível a pragas e doenças, é bem adaptada a uma grande variedade de situações climáticas, e normalmente não necessita de adubos. As culturas são resistentes ao inverno, o que permite a sua colheita nos meses frios. Podem ser cultivadas durante todo o ano, mas, na prática, existem duas épocas de cultivo, Primavera/Verão e Verão/Inverno, que afectam o crescimento e a composição química das plantas (12).

2.1.2. Valor alimentar da couve tronchuda

Entre muitos dos seus atributos saudáveis, os vegetais da família das crucíferas fornecem nutrientes importantes como as vitamina C e A, o ácido fólico, cálcio e potássio, fibras, tendo um baixo teor de gorduras e de calorias. Na Tabela 1 encontram-se os valores de composição nutricional da couve tronchuda e de outras couves habitualmente consumidas em Portugal, assim como os valores de ingestão diária recomendada de alguns nutrientes (para adultos).

Tabela 1. Valor nutricional (micro e macro nutrientes) da couve tronchuda cozida (por 100 g de parte edível)*.

	Tronchuda (folhas)	**Galega** (folhas)	**Grelos** (inflorescência)	**Brócolos** (inflorescência)	**R***
			Energia		
kcal	21	23	16	22	2490/2782[1a]
kJ	89	96	66	94	10500/11600[1a]
			Água (g)		
	92,1	90,5	93,7	91,9	
			Macronutrientes		
Proteína total (g)	2,1	2,1	1,8	2,8	56[1b]
Gordura total (g)	0,4	0,4	0,4	0,7	20-35[1c]
Hidratos carbono (g)	2,5	2,9	1,3	1,3	130[1c]
Sacarose (g)	0,2	0,2	0,2	0,1	
Amido (g)	0,1	0,4	0,1	0,1	
Oligossacáridos (g)	0	0	0,1	0,2	
Fibra alimentar (g)	2,4	2,7	2,2	2,3	38[1b]
			Ácidos gordos		
Saturados (g)	0	0,1	0,1	0,1	
Monoinsaturados (g)	0	0	0	0,1	
Polinsaturados (g)	0,3	0,3	0,2	0,3	
Trans (g)	0	0	0	0	
Ácido Linoleíco (g)	0,2	0,3	0,2	0,1	17[1b]
Colesterol (mg)	0	0	0	0	
Vitaminas					
A (eq. retinol) (μg)	207	362	174	114	900[5c]
Caroteno (μg)	1245	2172	838	687	
D (μg)	0	0	0	0	5[2b]
α-tocoferol (mg)	0,11	0,20	1,1	1,1	15[4c]
Tiamina (mg)	0,080	0,13	0,050	0,060	1,2[3c]
Riboflavina (mg)	0,050	0,070	0,040	0,040	1,3[3c]
Niacina (mg)	0,50	0,90	0,060	0,60	16[3c]
Triptofano/60 (mg)	0,30	0,50	0,030	0,50	
B6 (mg)	0,10	0,090	0,080	0,090	1,3[3c]
B12 (μg)	0	0	0	0	2,4[3c]
C (mg)	58	58	43	18	90[4c]
Folatos (μg)	46	38	54	47	400[3c]
Minerais					
Cinza (g)	0,51	1,40	0,60	0,97	
Na (mg)	108	127	108	101	1500[6b]
K (mg)	227	90	100	243	4700[6b]
Ca (mg)	71	264	106	56	1000[2b]
P (mg)	61	35	22	39	700[2c]
Mg (mg)	26	11	10	12	400[2c]
Fe (mg)	0,7	0,7	0,3	1,0	8[5c]
Zn (mg)	0,3	0,4	0,3	0,5	11[5c]

*Fontes: Tabela de composição de alimentos – Instituto Nacional de Saúde Dr. Ricardo Jorge; FAO/OMS: (1) Valores de referência para: energia, hidratos de carbono, fibra, gordura, ácidos gordos, colesterol, proteína, e aminoácidos (2002); (2) Valores de referência para: cálcio, fósforo, magnésio, vitamina D e fluoreto (1997); (3) Valores de referência para: tiamina, riboflavina, niacina, vitamina B_6, folato, vitamina B_{12}, ácido pantoténico, biotina e colina (1998); (4) Valores de referência para: vitamina C, vitamina E, selénio e carotenóides (2000); (5) Valores de referência para: vitamina A, vitamina K, arsénio, boro, crómio, cobre, iodo, ferro, manganês, molibdénio, níquel, silício, vanádio e zinco (2001); (6) Valores de referência para: água, potássio, sódio, cloreto e sulfato (2004).

R*. Referência; (a) Estimativa da energia necessária; (b) Ingestão adequada; (c) Valor máximo recomendado.

2.2. Caracterização do perfil metabólico

Sabe-se que as crúciferas são também uma fonte de compostos bioactivos como os compostos fenólicos (flavonóides e derivados do ácido cinâmico) e glucosinolatos (13). A couve tronchuda, em particular, foi previamente estudada relativamente aos seus teores de açúcares livres, proteína total, sais minerais e glucosinolatos (12, 14, 15).

2.2.1. Açúcares livres

Os açúcares solúveis são compostos do metabolismo primário, fornecendo energia e esqueletos de carbono para a síntese de outros compostos. Os açúcares podem também ser acumulados como resposta à falta de água, contribuindo para a osmoregulação, e como resposta a temperaturas muito baixas, uma vez que têm características crioprotectoras. Além disso, a presença de açúcares livres, sobretudo a glucose, afecta as características organolépticas da couve tronchuda (14).

A composição da couve tronchuda em frutose, glucose e sacarose foi estudada nas folhas de 4 cultivares. Nos extractos hidrometanólicos, o teor total de açúcares variou entre 102 e 159 mg/kg de peso seco, sendo que a frutose e a glucose, representaram mais de 87% do total. As quantidades totais e relativas variaram com a época de cultivo, predominando a glucose na época Primavera/Verão e a frutose na época Verão/Inverno (14).

2.2.2. Proteína e sais minerais

Rosa et Heaney avaliaram o teor de proteína e sais minerais (Ca, Mg, P, K, S, Fe, Mn e Zn) nas folhas internas e externas de couve tronchuda (12).

Verificou-se que o teor de proteína foi superior nas folhas internas (Tabela 2). Este resultado foi explicado pela tendência para acumulação de azoto nos tecidos jovens, que também recebem formas solúveis de azoto a partir das folhas mais velhas.

O teor médio de sais minerais nas folhas externas foi superior ao das folhas internas, com excepção do fósforo que tem tendência para se acumular no material vegetativo mais jovem (Tabela 2).

Em termos de época de cultivo, em geral a época Verão/Inverno origina concentrações superiores de sais minerais e proteína. Como neste caso o período de maior crescimento ocorre na estação menos favorável, o Inverno, a produção de

biomassa é menor e, consequentemente, a concentração de proteína e minerais maior.

Tabela 2 – Composição média de folhas externas e internas de couve tronchuda, expressa em matéria seca [adaptada de (12)].

Folhas	**Proteína** g/kg	**Ca** g/kg	**Mg** g/kg	**P** g/kg	**K** g/kg	**S** g/kg	**Fe** mg/kg	**Mn** mg/kg	**Zn** mg/kg
externas	170,9	35,5	2,7	4,4	24,7	8,3	100,6	80,9	47,6
internas	190,4	7,8	1,4	5,2	23,8	6,8	71,6	30,6	56,5

2.2.3. Glucosinolatos

A caracterização em glucosinolatos de vários tecidos da couve tronchuda foi realizada por Rosa et Heaney na década de 90 (12, 15). Foram identificados e quantificados 8 glucosinolatos diferentes, incluindo a sinigrina e a glucoiberina (Figura 4, Tabela 3). A sinigrina, que após hidrólise origina isotiocianato de alilo, é responsável pelo aroma pungente característico da mostarda e do rábano picante. A glucorafanina (que difere da glucoiberina por ter um grupo butilo em vez do grupo propilo) não foi detectada nesta variedade particular de *B. oleracea,* embora esteja descrita noutras crúciferas como os brócolos (16). Este glucosinolato assume uma particular importância uma vez que muitos dos estudos sobre os efeitos benéficos dos glucosinolatos são realizados com o sulforafano cujo precursor é a glucorafanina (17).

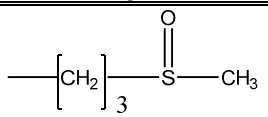
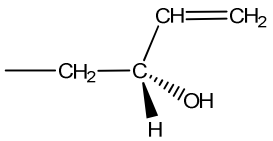
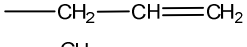
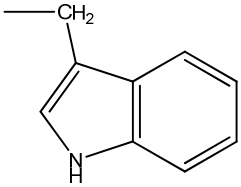
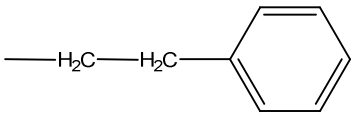
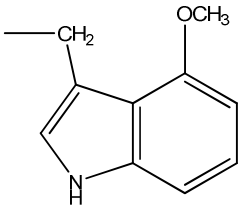
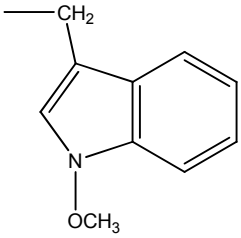
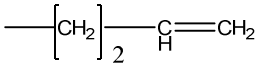
Glucosinolato	Grupo R
1 Glucoiberina (3-metilpropilsulfóxido glucosinolato)	
2 Progoitrina (2-Hidroxibut-3-enil glucosinolato)	
3 Sinigrina (Propen-2-enil glucosinolato)	
4 Glucobrassicina (Indol-3-ilmetil glucosinolato)	
5 Gluconasturtiina (Fenetil glucosinolato)	
6 Metoxiglucobrassicina (4-Metoxiindol-3-ilmetil glucosinolato)	
7 Neoglucobrassicina (l-Metoxiindol-3-ilmetil glucosinolato)	
8 Gluconapina (But-3-enil glucosinolato)	

Figura 4. Estruturas químicas dos grupos substituintes dos glucosinolatos descritos na couve tronchuda.

Na Tabela 3 encontram-se os teores médios de glucosinolatos de cada uma das matrizes estudadas. Os principais glucosinolatos encontrados foram a glucoiberina (representando 36,6%; 35,3% e 23,2% do total de glucosinolatos das folhas externas, folhas internas e inflorescências, respectivamente); a sinigrina (representando 19,5%; 26,5% e 31,1%), a glucobrassicina (representando 12,0%; 18,4% e 30,3%) e a 4-CH_3O glucobrassicina (representando 26,2%; 12,0% e 1,9%) do teor total em glucosinolatos.

Além disso verificou-se que a concentração de glucosinolatos é maior se as couves forem cultivadas na época Primavera/Verão e que a concentração total de glucosinolatos nas folhas internas é muito maior do que nas folhas externas,

provavelmente como resultado da acumulação de glucosinolatos nos tecidos jovens. A maior concentração de glucosinolatos foi encontrada nas inflorescências.

As quantidades de progoitrina, um glucosinolato considerado como um anti-nutriente que potencialmente origina a disfunção da tiróide, são reduzidas (variou entre 9,3 μmol/100 g de peso seco nas folhas externas e 151 μmol/100 g de peso seco nas inflorescências, representando menos de 2% nas folhas e menos de 6% do total de glucosinolatos nas inflorescências).

Tabela 3 – Concentração média de glucosinolatos na couve tronchuda, expressa em μmol/100 g matéria seca, nas folhas externas e internas (12) e nas inflorescências (15).

Compostos*	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Total**
F. externa	170,4	9,3	90,6	55,6	-	121,6	17,9	-	465
F. interna	689,2	35,6	518,3	360,3	3,1	234,3	196,1	8,2	1955
Inflorescências	583	151	783	764	132	48	36	22	2518

* Identidade dos compostos de acordo com a Figura 4.

2.2.4. Ácidos orgânicos

A natureza e concentração dos ácidos orgânicos são factores importantes que influenciam as características organolépticas de frutos e vegetais, nomeadamente o seu sabor. Alguns ácidos orgânicos são também antioxidantes muito eficientes.

As plantas, contrariamente aos animais e microrganismos, apresentam a capacidade de acumular ácidos orgânicos nos vacúolos celulares. A grande acumulação de ácidos orgânicos deve-se provavelmente ao seu papel na fotossíntese. Nas espécies com fotossíntese C_4 e metabolismo Crassulaceano (CAM, Crassulacean Acid Metabolism) os ácidos orgânicos são intermediários da assimilação de CO_2. Em plantas CAM, o CO_2 é absorvido à noite e incorporado no grupo carboxílico de um ácido orgânico (normalmente o ácido málico). Durante o foto-período seguinte, os ácidos orgânicos acumulados são descarboxilados para produzirem CO_2 que é consumido pelas reacções fotossintéticas do ciclo de redução do carbono. Este tipo de metabolismo dos ácidos orgânicos nas plantas CAM é muito importante, permitindo a sua sobrevivência em regiões áridas (18, 19).

Os ácidos orgânicos são importantes solutos de ajuste osmótico e servem para contrabalançar o excesso de catiões (20). A acumulação apoplástica dos ácidos orgânicos foi associada ao transporte de catiões nas raízes e tecidos vasculares das plantas. Alguns ácidos orgânicos servem de tampão ao excesso de catiões inorgânicos que existem em quantidades 4-17 vezes superiores aos aniões (21). Durante o crescimento vegetativo rápido, as taxas de redução do nitrato e a síntese de carboxilato e aminoácidos são elevadas. A redução de nitrato implica a formação de iões alcalinos tóxicos (1 mole de hidróxido por cada mole de nitrato reduzida). Como estes iões não podem ser eficientemente eliminados da célula, a planta sintetiza ácidos orgânicos, principalmente málico e citríco, para manter a homeostase (22).

Os ácidos orgânicos e compostos fenólicos presentes nos exsudados das raízes das plantas são importantes na solubilização de nutrientes, na restrição da passagem de metais tóxicos pela raiz e na atracção de microrganismos benéficos (23). A biossíntese, acumulação, transporte e exsudação dos ácidos orgânicos pelas raízes aumenta dramaticamente em resposta ao stress ambiental.

O metabolismo dos ácidos orgânicos é muito importante ao nível celular de muitas vias bioquímicas, incluindo a produção de energia e a formação de precursores para a biossíntese de aminoácidos e de metabolitos secundários, (Figura 5) (23).

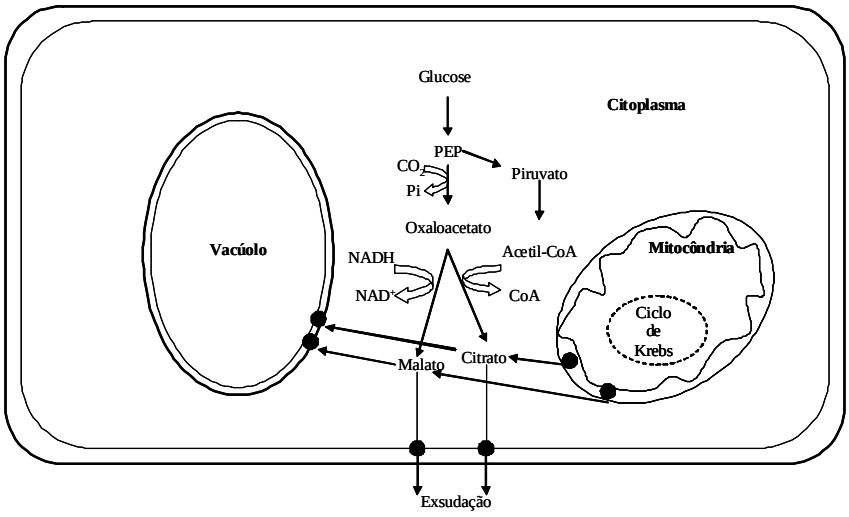


Figura 5. Fluxo de ácidos orgânicos numa célula vegetal [Adaptada de (23)].
CoA: Acetilcoenzima A, PEP: Fosfoenolpiruvato.

Os ácidos orgânicos mais comuns nas plantas são os ácidos tricarboxílicos do ciclo de Krebs (cítrico, aconítico, isocítrico, cetoglutárico, succínico, fumárico, málico e oxalacético). Destes, os ácidos málico, cítrico, aconítico e fumárico desempenham outras funções muito importantes no metabolismo das plantas. Os ácidos oxálico,

xiquímico, quínico e ascórbico também são muito importantes para as plantas. Na Figura 6 estão representadas as estruturas químicas de alguns ácidos orgânicos, com interesse para a definição do perfil metabólico em ácidos orgânicos da couve tronchuda, como se verá pelos resultados obtidos nos trabalhos realizados no âmbito desta tese.

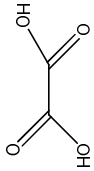
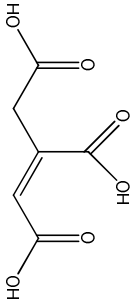
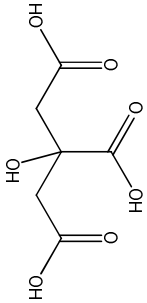
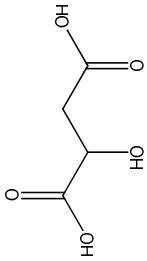
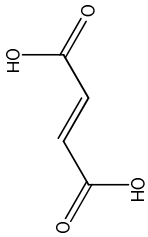
Ácido oxálico	Ácido aconítico	Ácido cítrico	Ácido málico	Ácido fumárico
				

Figura 6. Estrutura química de alguns ácidos orgânicos descritos na couve tronchuda.

2.2.5. Aminoácidos livres

Os aminoácidos são as unidades básicas de formação das proteínas, constituindo a principal forma de azoto nas plantas. Trata-se de uma classe de compostos biologicamente activos, existentes em frutos e vegetais, muito importantes na nutrição humana e que afectam a qualidade dos alimentos incluindo o sabor, o aroma e a cor (24).

A formação dos aminoácidos nas plantas inicia-se com a redução do nitrato absorvido pelas raízes, originando o amónio. A incorporação do amónio em esqueletos de carbono dá origem à glutamina e ao glutamato, que são as primeiras formas de azoto orgânico nas plantas. Por processos de transaminação o glutamato torna-se a principal fonte de grupos α-amina, necessários à formação da maior parte dos restantes aminoácidos, incluindo os 20 aminoácidos proteicos dos quais 8 (treonina, valina, isoleucina, leucina, triptofano, metionina, cisteína, fenilalanina e tirosina) são essenciais na alimentação humana (25).

Os aminoácidos estão envolvidos na síntese de praticamente todos os outros compostos de azoto das plantas incluindo aminas, alcalóides, heterósidos cianogénicos, glucosinolatos, porfirinas, purinas, piramidinas, citoquininas, auxinas, péptidos e proteínas.

A estrutura dos aminoácidos metionina, triptofano, tirosina e fenilalanina, precursores de muitos metabolitos secundários encontrados na couve tronchuda, está representada na Figura 7.

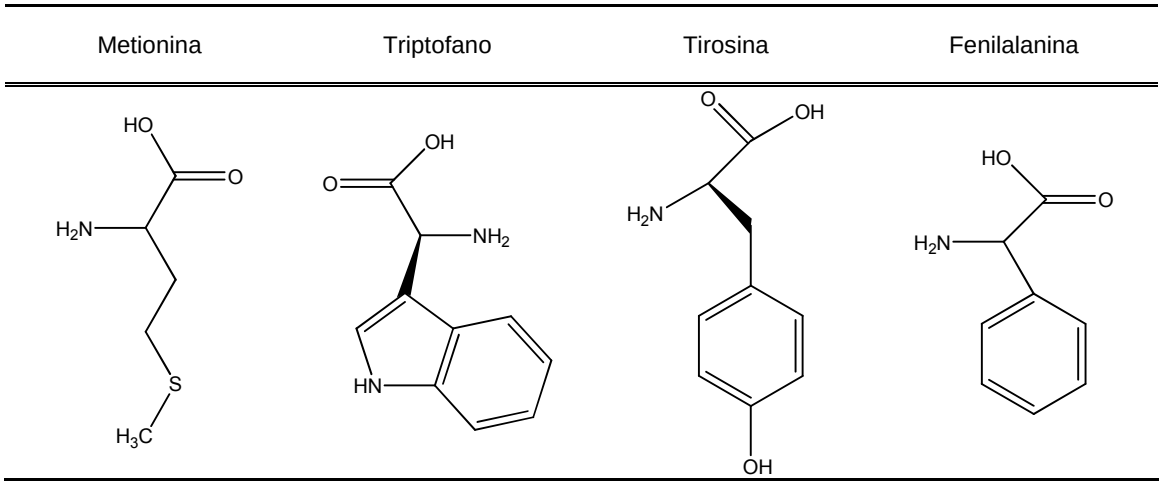


Figura 7. Estrutura de alguns aminoácidos usados no metabolismo secundário das plantas.

Além dos 20 aminoácidos proteicos, existem nas plantas mais de 300 aminoácidos raros não proteicos, sendo muitos deles tóxicos. Esses aminoácidos estão muito associados aos heterósidos cianogénicos e aos glucosinolatos. Nestas classes, os aminoácidos aparecem com ligações glicosídicas, sendo as geninas livres libertadas por hidrólise enzimática (26).

2.2.6. Compostos Voláteis

Os compostos voláteis, responsáveis pelos aromas das plantas, são moléculas de baixo peso molecular, em geral com baixo ponto de ebulição e pressões de vapor elevadas à temperatura ambiente. Para além destas características comuns, os diferentes grupos de compostos voláteis das plantas têm poucas semelhanças. Os principais grupos são os terpenóides, fenilpropanóides, derivados dos ácidos gordos e até alguns alcalóides, e contêm muitos grupos funcionais diferentes (ésteres, éteres, aldeídos, álcoois, alcenos, aminas, etc.).

Alguns compostos voláteis, são emitidos directamente para o ar, como ocorre nas flores, outros encontram-se na sua forma livre no interior dos tecidos sendo libertados quando as cutículas, paredes celulares ou membranas são rompidas e outros existem como conjugados, que originam moléculas voláteis por acção de enzimas durante o processamento como a maceração.

Apesar de não serem necessários para os processos metabólicos primários como a síntese proteica ou utilização directa de açúcares, muitos destes metabolitos são necessários à sobrevivência da planta, em funções de defesa da planta contra insectos herbívoros ou atracção de insectos que efectuem a polinização. Por exemplo, o eugenol é muito tóxico para insectos como os escaravelhos (27).

Os aromas dos alimentos estão directamente ligados às preferências alimentares e à palatibilidade. Muitos dos compostos que emanam aromas familiares, como o limoneno e o linalool, são comuns no reino vegetal. Outros como os isotiocinatos e compostos de enxofre resultantes da degradação dos glucosinolatos são restritos a um grupo específico, neste caso, as crucíferas.

As vias biossintéticas de muitos compostos voláteis das plantas foram rastreadas a compostos intermédios do metabolismo primário. Os hidratos de carbono, ácidos gordos e aminoácidos são as fontes de carbono mais importantes para os compostos responsáveis pelo aroma. A origem, as funções e algumas das propriedades dos compostos voláteis derivados dos glucosinolatos, e os terpenóides, metabolitos secundários com uma grande importância para o aroma das plantas encontram-se descritas nas secções seguintes.

2.2.6.1. Compostos voláteis derivados de glucosinolatos

Os glucosinolatos são metabolitos secundários das plantas que contêm azoto e enxofre conhecidos por serem os precursores dos compostos do aroma intenso característico das crucíferas (17). A sua estrutura (Figura 8) consiste num resíduo de β-D-glucopiranósido ligado por um átomo de enxofre a um éster de (Z)-N-hidroximinosulfato e um grupo R variável, derivado de um de oito aminoácidos.

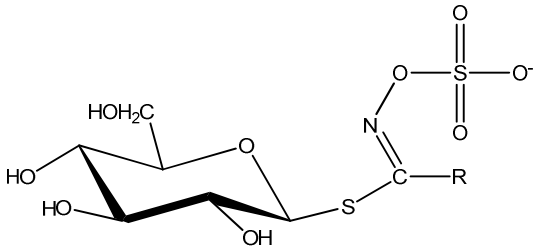


Figura 8. Estrutura química geral dos glucosinolatos.

Os glucosinolatos podem ser classificados em 3 categorias diferentes, de acordo com o aminoácido precursor: alifáticos se o aminoácido precursor for alanina, leucina, isoleucina, metionina ou valina; aromáticos se o aminoácido precursor for a

fenilalanina ou a tirosina e indólicos se o aminoácido precursor for o triptofano. Na maior parte dos glucosinolatos o aminoácido precursor do grupo R é extensivamente modificado estando descritos mais de 120 glucosinolatos. Os glucosinolatos alifáticos derivados da metionina são os que mais contribuem para a diversidade (17).

As plantas que acumulam glucosinolatos contêm sempre a enzima tioglucósido glucoidrolase conhecida como mirosinase, que hidrolisa a glucose do esqueleto principal (Figura 9). As mirosinases são o único grupo de β-tioglucosidases conhecido na natureza e usam apenas os glucosinolatos como substrato, não tendo qualquer actividade para outros *O*-glucósidos ou *S*-glucósidos *in vitro* (17). Os produtos resultantes da hidrólise são a glucose e uma genina instável que se rearranja para formar isotiocianatos, tiocianatos, nitrilos e outros produtos, dependendo do glucosinolato específico, isoenzima de mirosinase, pH da reacção e da presença de certos iões (Figura 9).

Em meio neutro, o rearranjo da genina origina geralmente um isotiocianato, mas se o meio for ligeiramente ácido e na presença de iões ferrosos, forma-se enxofre e nitrilos. Podem também formar-se tiocianatos, particularmente se a genina for um derivado do triptofano (29).

A hidrólise dos glucosinolatos nas plantas intactas é dificultada pela separação espacial da enzima ou pela inactivação da enzima. Os dois componentes misturam-se quando os tecidos são lesados (e.g. cortados, triturados ou mastigados) o que conduz à rápida formação dos produtos da hidrólise, incluindo os isotiocianatos, aos quais é atribuída a maior parte da actividade biológica dos glucosinolatos (17). Se a mirosinase for inactivada (e.g. pela cozedura) o metabolismo microbiano intestinal também contribui para a formação de isotiocianatos, embora em menor extensão (30). Pensa-se que as tioglucosidases intestinais apenas hidrolisam 10-20% dos glucosinolatos que passam no tracto digestivo (31).

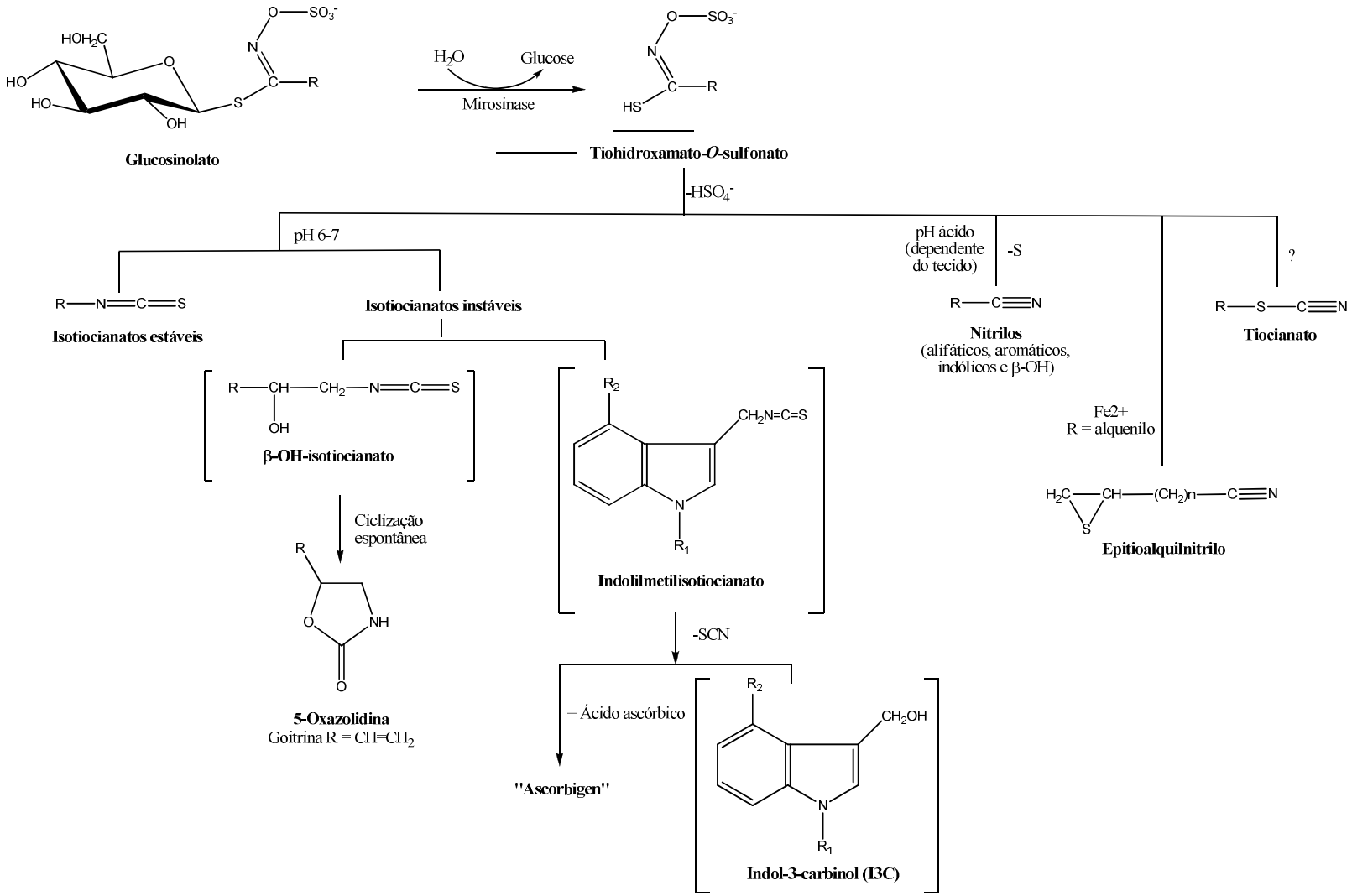


Figura 9. Hidrólise de glucosinolatos pela mirosinase e compostos obtidos após a degradação da molécula [adaptada de (28)].

A principal função dos glucosinolatos nas plantas é a defesa contra herbívoros e agentes patogénicos após a activação destes compostos durante a agressão à planta. Níveis elevados de produtos de hidrólise (especialmente nos rebentos) podem originar um sabor amargo desagradável (32). Além disso, os glucosinolatos em quantidades elevadas são tóxicos, inibem o crescimento e têm propriedades antinutricionais para uma grande variedade de inimigos potenciais das plantas, incluindo mamíferos, aves, insectos, moluscos, invertebrados aquáticos, nemátodes, bactérias e fungos. Não se conhece o mecanismo pelo qual os glucosinolatos exercem o seu efeito tóxico, mas sabe-se que os isotiocianatos têm tendência para reagir com os grupos amino e sulfidrilo das proteínas, *in vitro* (17).

Os glucosinolatos também podem atrair herbívoros que se adaptaram, como as lagartas da couve da espécie *Pieris brassicae.* Estas têm a capacidade de redireccionar a hidrólise dos glucosinolatos catalizada pela mirosinase para a formação de nitrilos em vez de isotiocianatos, sendo os nitrilos formados excretados nas fezes (33).

Numerosos estudos epidemiológicos sugerem que o consumo de vegetais da família das crucíferas está inversamente relacionado com a incidência de diversas formas de cancro, particularmente o cancro colorectal (34). Alguns isotiocianatos foram identificados como potentes indutores de enzimas de biotransformação de fase II, incluindo a quinona redutase (QR) e as transferases da glutationa (GST) que estão associadas à diminuição da susceptibilidade à carcinogénese química (30, 31).

Alguns isotiocianatos induzem enzimas de fase I, outros só de fase II e alguns induzem ambas. Geralmente, os compostos que induzem as enzimas de fase I (através do XRE (Xenobiotic Response Element) e de fase II (através do ARE (Antioxidant Response Element) contribuem para a eliminação dos compostos carcinogénicos, enquanto que os que só induzem as enzimas de fase I, podem acelerar, em vez de retardar os carcinomas com origem em compostos químicos (30). Para além de induzir enzimas de fase II, pensa-se que isotiocianatos como o sulforafano (isotiocianato presente nos brócolos derivado do 4-metilbutilsulfóxido glucosinolato) podem prevenir o crescimento de tumores, bloqueando o ciclo celular e promovendo a apoptose (17). Os glucosinolatos também podem potenciar as defesas antioxidantes e assim aumentar a resistência às espécies reactivas de oxigénio (31).

Apesar dos efeitos benéficos reconhecidos, os glucosinolatos podem ser prejudiciais, sobretudo em pessoas com disfunção da tiróide. Quando a cadeia lateral dos glucosinolatos tem um grupo hidroxilo no carbono 2, os isotiocianatos formados são instáveis e ciclizam a oxazolidina-2-tionas, uma classe de substâncias conhecida

por provocar o bócio (17). As oxazolidina-2-tionas inibem a função da tiróide bloqueando a incorporação do iodo nos precursores da tiroxina e suprimindo a libertação pela tiróide desta hormona. Contudo, os efeitos adversos dos produtos da hidrólise dos glucosinolatos só se fazem sentir para concentrações muito superiores às obtidas com o consumo normal de crucíferas, não existindo perigo com o consumo normal.

2.2.6.2. Compostos voláteis derivados de terpenóides

Os isoprenóides, terpenos ou terpenóides são os compostos mais abundantes e estruturalmente mais diversos que existem na natureza, primariamente como parte dos óleos essenciais das plantas. Nas plantas, a função dos terpenóides é muito diversificada, actuando como estímulo para os insectos envolvidos na polinização, repelentes para predadores, agentes antimicrobianos, especialmente contra fungos, pigmentos acessórios para a fotossíntese (carotenóides) e factores de crescimento. Muitos dos terpenóides não são voláteis e estão envolvidos em processos como a estrutura da membrana (esteróides), fotossíntese (cadeias laterais da clorofila, carotenóides) e regulação do crescimento (giberelinas, ácido abcísico e brassinosteróides). Os terpenóides voláteis (hemiterpenóides, monoterpenóides, sesquiterpenóides e alguns diterpenóides) estão envolvidos nas interacções entre plantas e insectos herbívoros e polinizadores e nas respostas gerais de defesa e ao stress. Muitos terpenóides têm propriedades antiretrovirais e antimaláricas (35).

Os isoprenóides são classificados de acordo com o número de unidades básicas de isopreno ($CH_2=C(CH_3)-CH=CH_2$) em hemiterpenóides (n=5), monoterpenóides (n=10), sesquiterpenóides (n=15), diterpenóides (n=20), triterpenóides (n=30), tetraterpenóides (n=40, carotenóides) e politerpenóides (n>40) (36). Os sesquiterpenóides são a classe que mais contribui para a variedade de odores (27).

Os isoprenóides são enzimáticamente sintetizados *de novo* a partir de acetil CoA e piruvato. Apesar da sua diversidade, todos os terpenóides derivam de duas unidades básicas comuns de cinco carbonos, o isopentenilo difosfato (IPP) e o seu isómero dimetilalil difosfato (DMADP), a partir dos quais todos os outros isoprenóides são formados por adição cabeça-cauda. Estas unidades são sintetizadas em duas vias paralelas: a via acetato/mevalonato (MVA), activa no citosol, e a via da 1-desoxi-D-xilulose fosfato (DOXP), activa nos plastídios (37). De uma forma geral, a via acetato/mevalonato é a via de biossíntese de esteróis, sesquiterpenos e triterpenóides, enquanto que a via da 1-desoxi-D-xilulose fosfato é a via de biossíntese de

carotenóides, fitol (cadeia lateral das clorofilas), plastoquinonas, isopreno, monoterpenos e diterpenos (38). Os precursores directos dos isoprenóides, os compostos de cadeia linear difosfato de geranilo (GDP, C_{10}), difosfato de farnesilo (FDP, C_{15}) e difosfato de geranilgeranilo (GGDP, C_{20}) são produzidos pelas actividades de três prenil transferases, que catalisam a adição de unidades IDP a difosfatos de prenilo com uma dupla ligação alílica ao grupo difosfato. A conversão dos vários difosfatos de prenilo em monoterpenos, sesquiterpenos e diterpenos é catalisada pelas enzimas terpeno sintetases. Os triterpenos, esteróides e tetraterpenos derivam da condensação de duas moléculas de FDP e GGDP (Figura 10) (39).

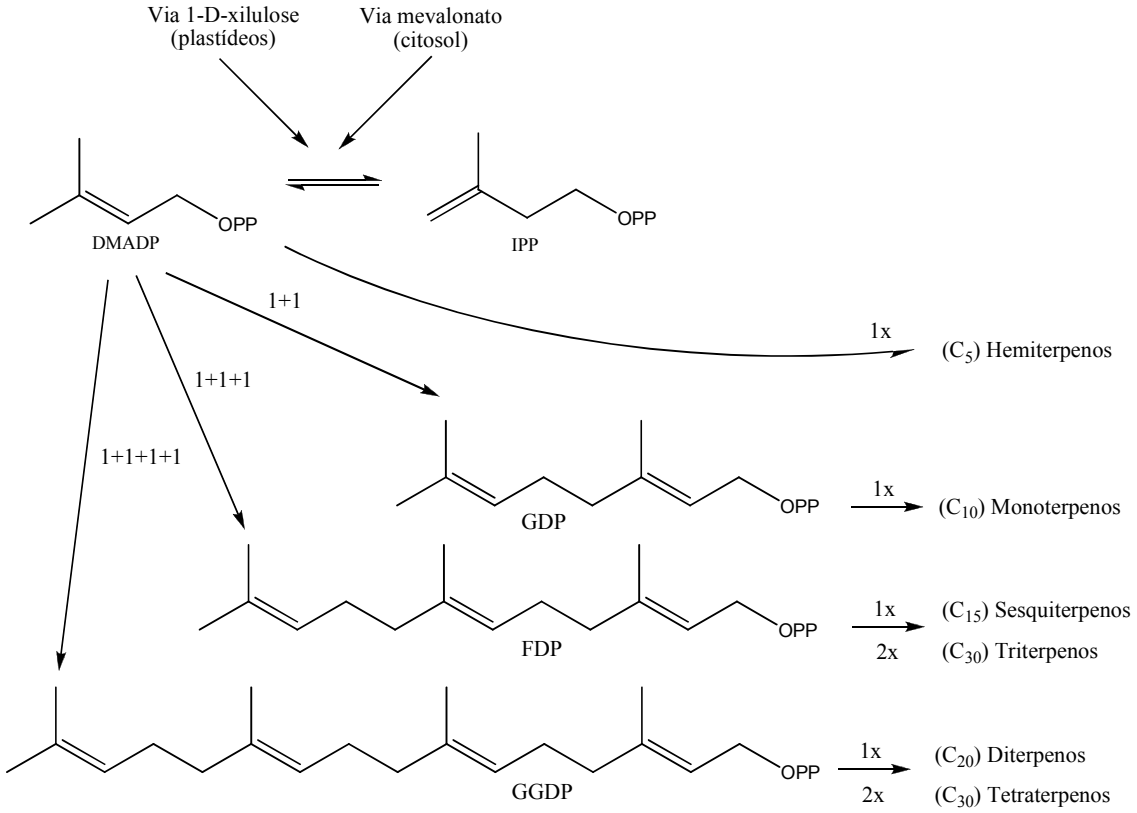


Figura 10. Esquema geral da biossíntese de terpenos [adaptada de (39)].

DMADP: dimetilalil difosfato; FDP: difosfato de farnesilo; GDP: difosfato de geranilo; GGDP: difosfato de geranilgeranilo; IPP: isopentenilo difosfato.

Os carotenóides são os pigmentos naturais de maior distribuição na natureza, variando do amarelo claro ao vermelho escuro. Além de serem pigmentos auxiliares na fotossíntese e corantes, os carotenóides são os precursores dos apocarotenóides como a fito-hormona ácido absícico, as moléculas de sinalização retinal e ácido retinóico e compostos voláteis como a β-ionona. Os carotenóides têm um esqueleto com 8 unidades isoprénicas, agrupadas em duas unidades de 20 carbonos,

condensadas cabeça com cabeça, formando uma longa cadeia de duplas ligações conjugadas. A formação de apocarotenóides pela quebra oxidativa dos carotenóides origina os precursores de diferentes compostos, inicialmente aldeídos ou cetonas nos locais da quebra, que podem sofrer uma “remodelação oxidativa” (Figura 11).

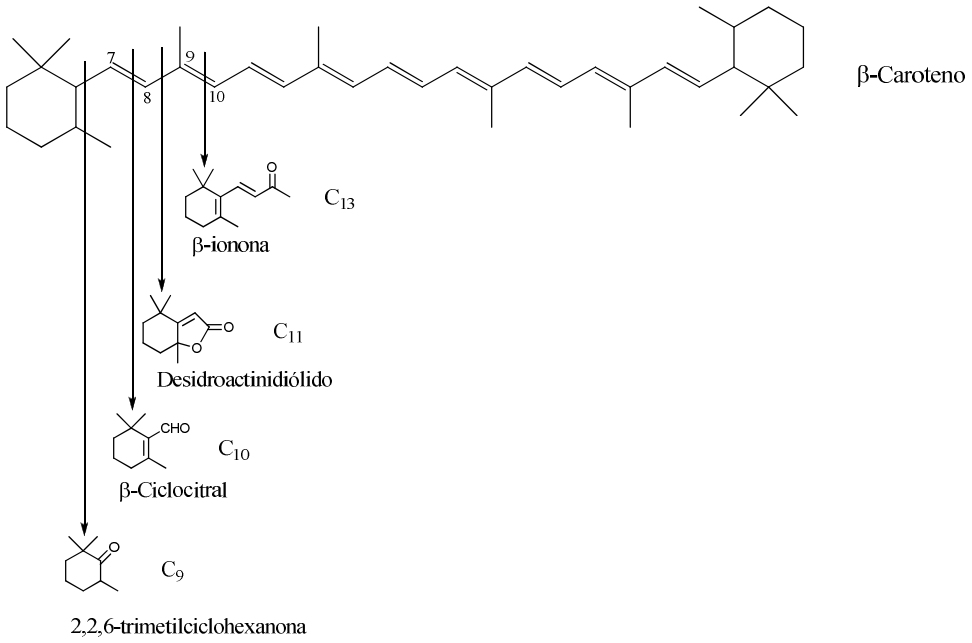


Figura 11. Produtos da quebra oxidativa do β-caroteno [adaptada de (36)].

Tal como as moléculas parentais, os apocarotenóides são isoprenóides não-polares com um sistema de duplas ligações extendido. Alguns apocarotenóides poderão resultar da síntese directa a partir de moléculas mais pequenas, mas a maioria tem origem na quebra oxidativa de isoprenóides maiores (40). A quebra dos carotenóides pode ser simétrica ou assimétrica e pode acontecer por mecanismos enzimáticos (envolvendo enzimas conhecidas como dioxigenases de quebra de carotenóides (CCD’s)) ou não enzimáticos. A diversidade destes compostos resulta do tipo de carotenóide precursor, do local da quebra e da remodelação oxidativa (36).

2.2.7. Compostos polifenólicos

A maioria dos compostos polifenólicos naturais é produzida por plantas superiores. São conhecidas mais de 8000 estruturas de polifenóis, tendo em comum um anel aromático com pelo menos um hidroxilo substituinte. Nas plantas os polifenóis desempenham uma vasta gama de funções, estando envolvidos no seu

desenvolvimento e em interacções com o ambiente. Por exemplo, os estilbenos, cumarinas e isoflavonóides são fitoalexinas, os flavonóides protegem contra as radiações UV e participam na sinalização e interacção com simbiontes e a acetosiringona e o ácido salicílico estão envolvidos nas interacções planta-agente patogénico.

Os efeitos benéficos dos compostos polifenólicos para os seres humanos são atribuídos às suas propriedades antioxidantes (41), acção estrogénica (42) e a um largo espectro de actividades antimicrobianas e farmacológicas (antiinflamatórias, antialérgicas e vasodilatadoras) (43-46). Destas, a propriedade melhor documentada é a sua capacidade para agir como antioxidantes (47).

A biossíntese dos ácidos cinâmicos e os flavonóides, duas classes de compostos polifenólicos muito importantes nas plantas do género *Brassica*, particularmente na espécie *B. oleracea* a que pertence a couve tronchuda encontram-se descritas nas secções seguintes. A biossíntese e degradação da sinapoilcolina, que pode ser considerada um biomarcador importante na assinatura metabólica do género *Brassica*, e que se encontra sobretudo nas sementes, que também foram objecto de estudo desta tese, também é descrita.

2.2.7.1. Ácidos cinâmicos

Os ácidos cinâmicos são fenilpropanóides (C_6-C_3), contendo além do hidroxilo fenólico uma função carboxilo e uma dupla ligação na cadeia lateral. A dupla ligação possibilita a existência de 2 isómeros *cis e trans,* sendo que os isómeros *trans* mais estáveis predominam na natureza. Estes compostos variam no padrão de substituição do anel aromático.

Os ácidos cinâmicos são habitualmente encontrados nas plantas na sua forma conjugada, muitas vezes como grupos acilo de heterósidos flavonóidicos. A ligação aos açúcares (ou heterósidos flavonóidicos) é facilmente quebrada com uma hidrólise alcalina, o que permite a identificação dos ácidos por LC-DAD (liquid chromatography with a diode array detector) (48).

Os ácidos cinâmicos são biossintetizados nas plantas pela via xiquimato como se indica na Figura 12. O ácido xíquimico é um precursor dos aminoácidos aromáticos fenilalanina e tirosina, de compostos indólicos incluindo o aminoácido triptofano, de alcalóides e de compostos fenólicos.

O ácido xiquímico resulta da condensação de uma unidade de eritrose-4-fosfato com uma unidade de fosfoenolpiruvato para formar 3-desoxi-arabinoheptulsonato-7-fosfato (DAHP), numa reacção catalizada pela enzima DAHP

sintetase (enzima 1, Figura 12) (49). O grupo fosfato actua como uma base interna para promover a sua própria eliminação. A redução da cetona então formada com NADH e a eliminação de um protão permite a abertura do anel que, ao voltar a ciclizar, dá origem ao 3-desidroquinato (enzima 2) (50). Apesar de esta reacção necessitar de NAD como cofactor, o mecanismo enzimático regenera-o. O 3-desidroquinato é transformado em 3-desidroxiquimato pela enzima desidroquinase desidratase (enzima 3) (51). A redução a ácido xiquímico é catalisada pela xiquimato desidrogenase (enzima 4) que usa NADPH como cofactor.

Na biossíntese do corismato, a 5-enolpiruvoilxiquimato 3-fosfato sintetase ou EPSP sintetase (enzima 6) cataliza a transferência do resíduo de enolpiruvoilo do fosfoenolpiruvato (PEP) para o xiquimato 3-fosfato formando EPSP e fosfato inorgânico (52, 53). A corismato sintetase (enzima 7) catalisa a conversão do EPSP em corismato (54).

A corismato mutase é responsável pela conversão do corismato em isocorismato (ou prefenato) (55). O prefenato é convertido pela prefenato desidratase (enzima 9) em fenilpiruvato (55, 56). Alternativamente, na presença da enzima prefenato desidrogenase (prefenato NAD-oxiredutase) o produto obtido é o *p*-hidroxifenilpiruvato. A enzima aminotransferase aromática (ou aminoácido aromático transaminase; enzima 10) catalisa a transaminação do glutamato para um oxo-ácido aromático, obtendo-se o aminoácido aromático L-fenilalanina e 2-oxoglutarato (57).

A fenilalanina é o precursor dos ácidos cinâmicos na via geral dos fenilpropanóides. Esta via liga o metabolismo primário com a biossíntese de fenilpropanóides nas plantas pela acção sequencial da fenilalanina amónia liase (PAL; enzima 11), cinamato 4-hidroxilase (CA4H; enzima 12) e 4-cumarato:coenzima A liase (4CL; enzimas 13 e 18).

A PAL desempenha um papel chave na sequência biossintética dos fenilpropanóides, catalisando a biotransformação da L-fenilalanina em ácido trans-cinâmico e amónia (58, 59). A PAL é específica para a L-fenilalanina embora o L-triptofano também seja um substracto. Os outros aminoácidos comuns não são desaminados. O ácido cinâmico é posteriormente modificado pela acção de hidroxilases e *O*-metiltransferases, levando à síntese dos vários ácidos hidroxicinâmicos.

A cinamato 4-hidroxilase é uma enzima do citocromo P450 que catalisa o primeiro passo de oxigenação da via dos fenilpropanóides nas plantas superiores: a hidroxilação do ácido *trans*-cinâmico em ácido *trans p*-cumárico. Esta enzima é induzida pela luz, e está envolvida na síntese de compostos de defesa, na cicatrização e no combate a infecções (60).

A enzima *p*-cumarato (hidroxicinamato):coenzima A Liase catalisa a conversão do *p*-cumarato e de outros *p*-hidroxicinamatos nos respectivos tioésteres de CoA. Os ésteres activados são usados em muitas vias biossintéticas incluindo a biossíntese de lenhina, lenhanas, suberina, flavonóides e vários compostos fenólicos de baixo peso molecular (61). A 4CL de várias espécies de plantas aceita *p*-cumarato, ferulato e cafeato como substratos em ordem decrescente de preferência, mas não aceita o sinapato. Assim, é possível que o sinapoil-CoA se forme por uma via alternativa envolvendo a cafeoil-CoA *O*-metiltransferase, que produz feruloil-CoA, o substrato da cinamoil-CoA redutase, a primeira enzima dedicada à síntese de monolenhóis (62).

A hidroxilação do ácido *p*-cumárico pela *p*-cumarato-3-hidroxilase (enzima 14) origina o ácido cafeico. O ácido cafeico activado pode ser metoxilado a ácido ferúlico pela cafeoil *O*-metiltransferase (CCOMT; enzima 15) (63). A feruloil 5-hidroxilase (F5H; enzima 16) catalisa a hidroxilação do ácido ferúlico a ácido 5-hidroxiferúlico. Tal como a CA4H, as enzimas F5H são monooxigenases do grupo do citocromo P450, caracterizadas por conter grupos heme, sendo dependentes do NADPH e de oxigénio (64). A 5-hidroxiferulato *O*-metiltransferase (enzima 17) catalisa a conversão do ácido 5-hidroxiferúlico em ácido sinápico. A *O*-metilação do ácido cafeico a ácido ferúlico e do ácido 5-hidroferúlico a ácido sinápico pode ser induzida por agentes patogénicos (65).

Nas plantas, uma das principais funções dos ácidos hidroxinâmicos é a sua utilização como blocos para a biossíntese da lenhina. Por exemplo, o *p*-cumarato:CoA pode ser convertido em *p*-cumaraldeído sendo a reacção catalisada pela hidroxicinamil coenzima A redutase (enzima 19). Este pode por sua vez ser convertido em *p*-cumaril álcool (hidroxicinamil álcool desidrogenase (enzima 20) que na presença de peroxidases (enzima 21) levam a formação da lenhina (65, 66).

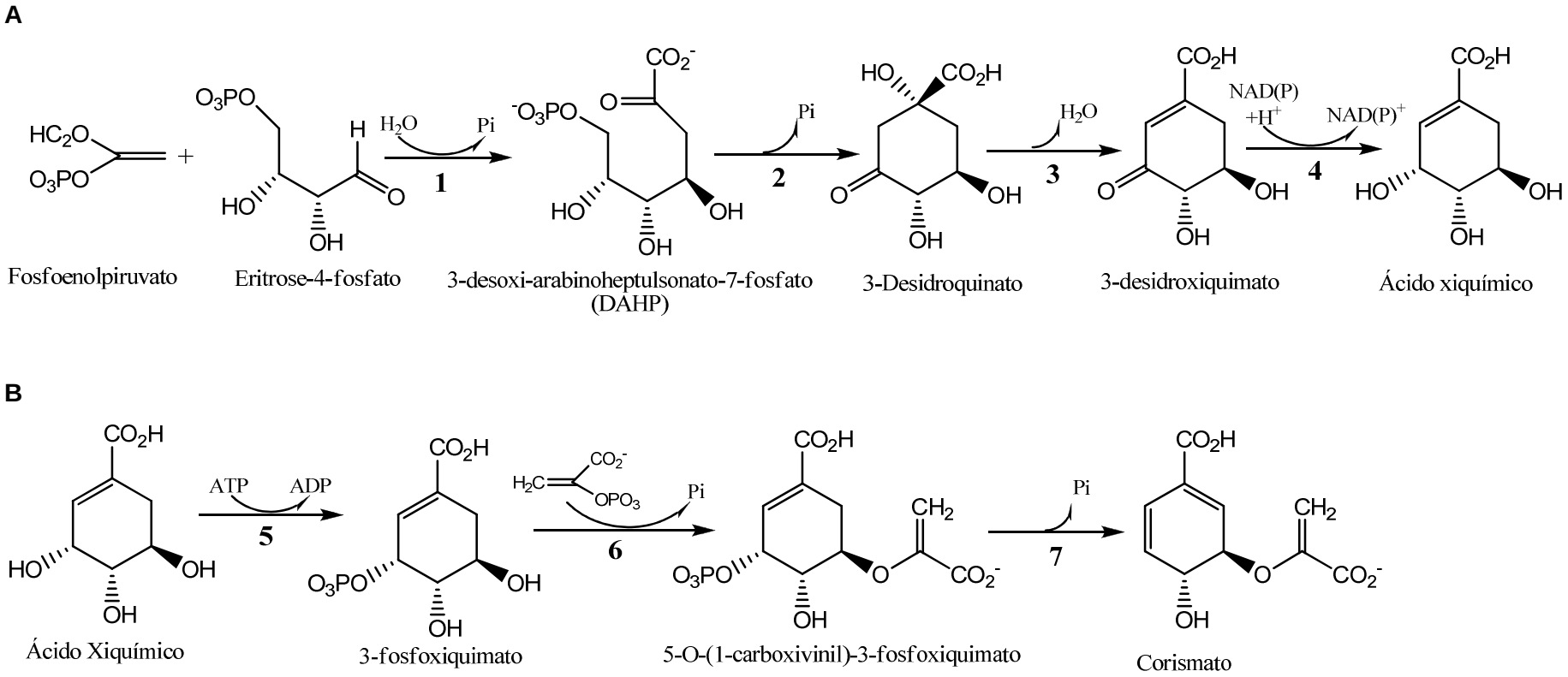


Figura 12. Via geral dos fenilpropanóides. A. Biossíntese do Ácido Xiquímico; B. Biossíntese do Corismato

Enzimas: 1: DAHP Sintetase; 2: Desidroquinato sintetase; 3: Desidroquinase; 4: Xiquimato desidrogenase; 5: Xiquimato cinase; 6: 3-Fosfoxiquimato 1-carboxiviniltransferase; 7: Corismato Sintetase; 8: Corismato mutase.

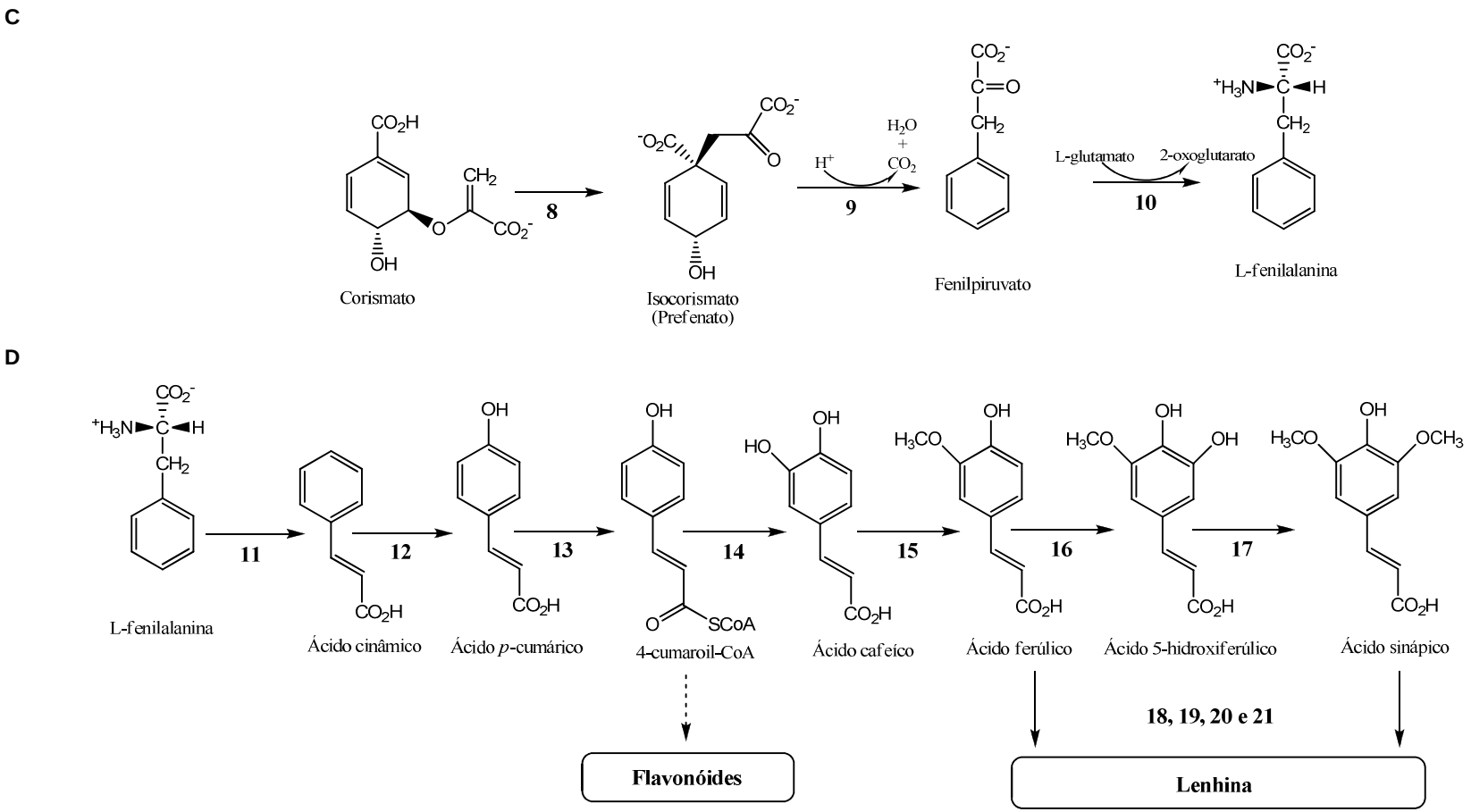


Figura 12. Via geral dos fenilpropanóides. C. Biossíntese da Fenilalanina; D. Biossíntese dos ácidos hidroxicinâmicos. (cont.).

Enzimas: 8: Corismato mutase; 9: Prefenato desidratase; 10: Aminoácido aromático transaminase; 11 (PAL): Fenilalanina Amónia Liase; 12 (CA4H): Cinamato 4-hidroxilase; 13 (4CL) e 18. *p*-Cumarato (hidroxicinamato) : coenzima A Liase; 14: *p*-Cumarato-3-hidroxilase; 15 (CCOMT) e 17: Cafeato/5-hidroxiferulato *O*-metiltransferase; 16 (F5H). Ferulato-5-hidroxilase; 19: Hidroxicinamil coenzima A redutase; 20: Hidroxicinamil álcool desidrogenase, 21: Peroxidase

Os ácidos cinâmicos também aparecem muitas vezes conjugados com o ácido quínico, constituindo o sub-grupo dos ácidos clorogénicos. Na biossíntese dos ácidos clorogénicos (Figura 13) está envolvida a enzima hidroxicinamoiltransferase (HCT) que faz a esterificação do ácido quínico com o ácido *p*-cumárico activado com coenzima A. O resíduo *p*-cumaroílo pode posteriormente ser modificado, originando outros ácidos hidroxicinâmicos. Por exemplo, a enzima *p*-cumaroato 3-hidroxilase (C3H) catalisa a formação do cafeoilquinato a partir de *p*-cumaroilquinato (66).

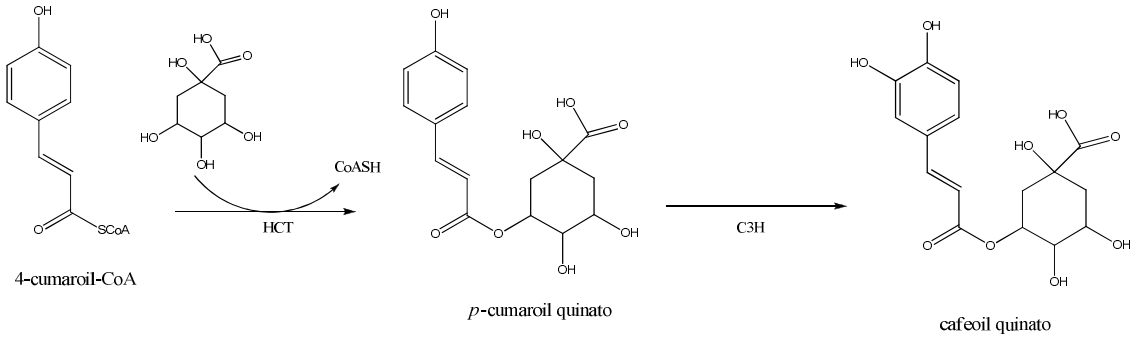


Figura 13. Biossíntese dos ácidos clorogénicos.

HCT: Hidroxicinamoiltransferase; C3H: p-cumaroato 3-hidroxilase; CoASH: coenzima A

2.2.7.2. Sinapoilcolina

A sinapoilcolina, um éster de ácido sinápico com a colina, acumula-se nas sementes da maior parte das plantas do género *Brassica,* sendo usada como marcador quimiotaxonómico deste género (67). O teor de ésteres de colina (sinapinas) e também de glucosinolatos pode inviabilizar a utilização destas plantas (sobretudo das sementes) na alimentação humana e animal. Os ésteres de colina são hidrolisados durante a digestão e a colina é degradada a trimetilamina, o que confere um aroma desagradável. A biossíntese e degradação da sinapina encontram-se esquematizadas na Figura 14 (68).

O sinapato proveniente da via dos fenilpropanóides é conjugado com a colina durante o desenvolvimento da semente. Quando a semente germina dá-se a hidrólise da sinapoilcolina, libertando-se o sinapato que é novamente conjugado com malato no rebento. O éster de sinapoilglucose (éster β-acetálico) é o dador de sinapato na síntese da sinapina catalisada pela 1-O-sinapoilglucose:colina sinapoiltransferase (SCT), sendo também usado na síntese de sinapoilmalato pela 1-O-sinapoilmalato:colina sinapoiltransferase (SMT). Este dador rico em energia é formado pela sinapato glusosiltransferase (SGT).

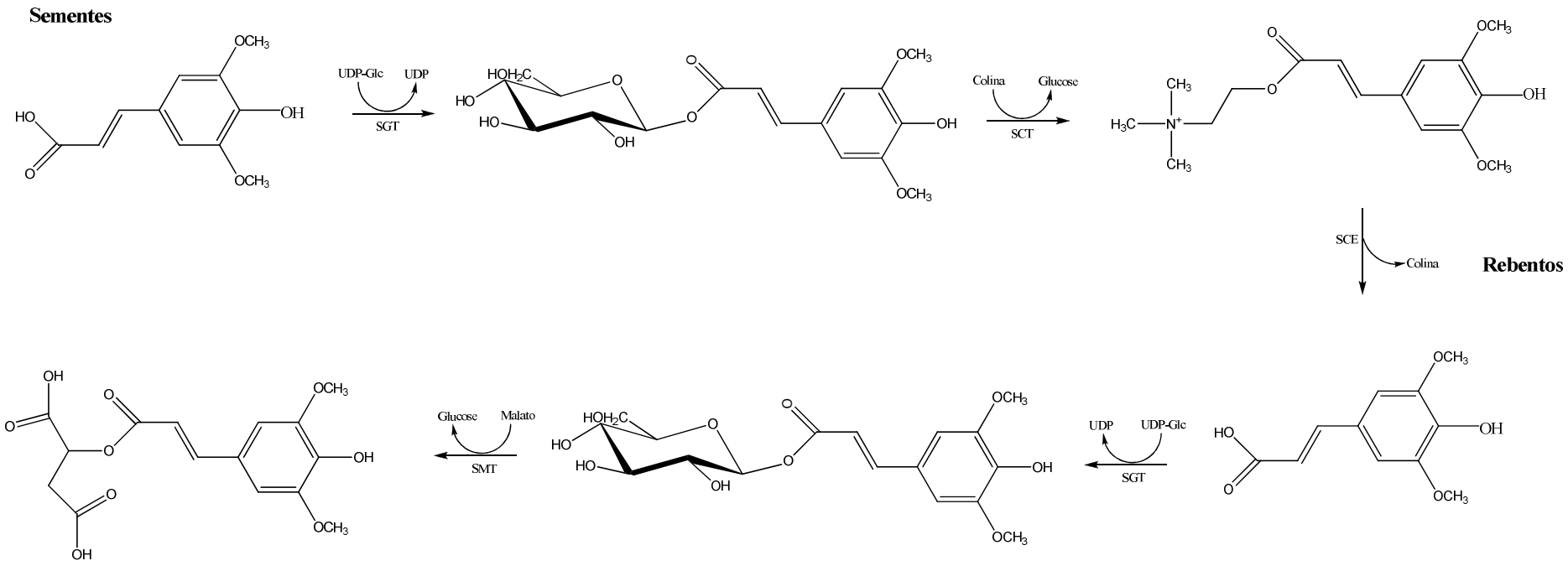


Figura 14. Biossíntese de três dos principais ésteres de ácido sinápico em sementes e rebentos de crucíferas [adaptada de (68)].

SGT: Sinapato glusosiltransferase; SCT: 1-*O*-sinapoilglucose:colina sinapoiltransferase; SCE: Sinapina esterase; SMT: 1-*O*-sinapoilmalato:colina sinapoiltransferase.

2.2.7.3. Flavonóides

Os flavonóides constituem uma das maiores classes de compostos naturais estimando-se que cerca de 2% do carbono fotossintetizado seja convertido pelas plantas em flavonóides ou compostos relacionados (48).

Os flavonóides são difenilpropanos (C_6-C_3-C_6) constituídos por dois anéis aromáticos ligados por uma cadeia de 3 carbonos (Figura 15).

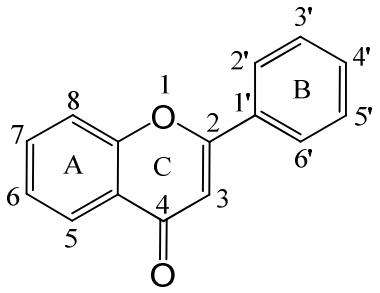


Figura 15. Estrutura química e numeração de flavonóides.

Os flavonóides podem ser sub-divididos em 9 sub-grupos principais, com base na oxidação do anel heterocíclico C: chalconas, flavonas, flavonóis, flavanonas, desidroflavonóis, flavan-3,4-dióis, catequinas, antocianidinas e isoflavonas (Figura 16). O sub-grupo das auronas não tem uma distribuição tão generalizada, mas também se encontra com alguma frequência.

Como existe uma grande tendência para que as plantas quimiotaxonomicamente relacionadas produzam tipos semelhantes de flavonóides, estes são habitualmente usados como marcadores taxonómicos (47).

As características mais importantes para a definição das diferentes classes de flavonóides são o nível de oxigenação e o local de ligação ao anel B (flavonóides ou isoflavonóides). Dentro de cada classe a “ornamentação” da estrutura base com grupos glicosilo, carboxilo, metilo e hidroxilo origina uma grande variedade de compostos (69). A esteroquímica, posição e natureza das substituições e o grau de polimerização e ligações entre as unidades básicas também contribuem para essa variedade (70).

Tal como os ácidos cinâmicos, os flavonóides existem habitualmente na forma glicosilada. Nos heterósidos, os açúcares podem estar ligados aos grupos hidroxilo ou directamente ao carbono de um anel aromático (*O* ou *C*-glicosilação respectivamente)

(71). A componente glicosídica pode variar muito no número e tipo de açúcares e na configuração do(s) carbono(s) anomérico(s) das unidades glícosidicas (72).

O campferol e a quercetina são os flavonóis mais comuns no reino vegetal, e juntamente com a isoramnetina (e as antocianinas na couve roxa) são os únicos flavonóides já identificados em espécies de *B. oleracea*. A semelhança das suas estruturas contrasta com a grande diversidade de número, posição e estrutura de glicósidos e grupos acilo ligados a estas geninas (47).

2.2.7.4. Biossíntese de flavonóides

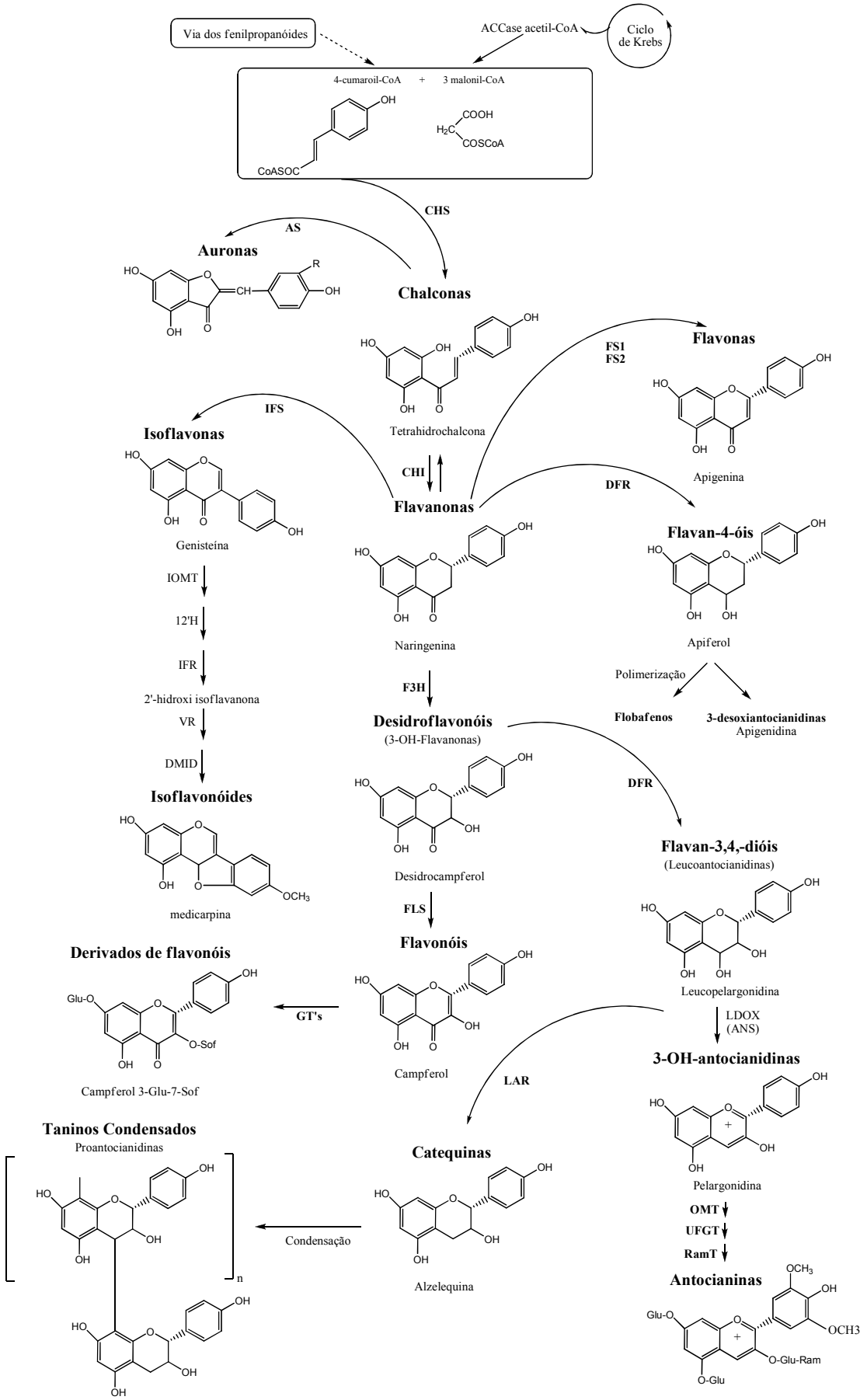
Com poucas excepções, só as plantas possuem a capacidade de biossintetizar flavonóides usando duas vias biossintéticas principais: a via acetato e a via xiquimato. Biogeneticamente o anel A tem origem numa molécula de resorcinol ou floroglucinol sintetizado na via acetato, e tem um padrão de hidroxilação característico nas posições 5 e 7. O anel B tem origem na via xiquimato, é geralmente hidroxilado nos carbonos 4’ ou 3’, 4’ ou ainda 3’, 4’ e 5’.

A via biossintética dos flavonóides é um dos sistemas metabólicos mais estudados nas plantas e parece ser uma grande e complexa rede metabólica rigorosamente orquestrada (73). Esta via encontra-se esquematizada na Figura 16.

Figura 16. Esquema das principais ramificações da biossíntese de flavonóides (chalconas, auronas, isoflavonóides, flavonas, flavonóis, flavanodióis, antocianinas, catequinas, taninos condensados e flobafenos) [adaptada de (70, 73)].

ACCase acetil-CoA: acetil CoA carboxilase; ANS: antocianidina sintetase; AS: aureusidina sintetase; CHS: chalcona sintetase; CHI: Chalcona isomerase; DFR: desidroflavonol 4-redutase; DMID: 7,2’-dihidroxi, 4’-metoxiisoflavanol desidratase; F3H: flavanona 3-hidroxilase; FLS: flavonol sintetase; FS1/FS2: flavona sintetase; I2’H: isoflavona 2’-hidroxilase; IFR: isoflavona redutase; IFS: isoflavona sintetase; IOMT: isoflavona *O*-metiltransferase; LAR: leucoantocianidina redutase; LDOX: leucoantocianidina dioxigenase; OMT: *O*-metiltransferase; RamT: ramnosilo transferase; UFGT: UDP flavonóide glucosilo transferase; VR: vestitona redutase

Nota: As enzimas flavonóide 3’-hidroxilase (F3’H) e flavonóide 3’5’-hidroxilase (F3’5’H), que catalisam a hidroxilação do anel B, não estão representadas.



(legenda na página 33)

A maioria das enzimas da biossíntese de flavonóides pertence a três classes de enzimas encontradas em todos os organismos: dioxigenases dependentes do 2-oxoglutarato, redutases dependentes do NADPH e hidroxilases do citocromo P450. As dioxigenases dependentes do 2-oxoglutarato são a flavanona 3-hidroxilase (F3H), a flavonol sintetase (FLS), a flavona sintetase (FS1) e a leucoantocianidina dioxigenase (LDOX). As redutases dependentes do NADPH são a desidroflavonol 4-redutase (DFR), a leucoantocianidina redutase (LAR), a isoflavona redutase (IFR) e a vestitona redutase (VR). As hidroxilases do citocromo P450 são a flavonóide 3'-hidroxilase (F3'H), a flavonóide 3'5'-hidroxilase (F3'5'H) a isoflavona sintetase (IFS), a isoflavona 2'-hidroxilase (I2'H) e a flavona sintetase (FS2). Pelo contrário, as enzimas chalcona sintetase (CHS) e particularmente a chalcona isomerase (CHI) envolvidas na biossíntese das chalconas e das flavanonas parecem ser exclusivas das plantas (73).

Na biossíntese dos flavonóis, o primeiro passo específico é catalisado pela CHS, na reacção de condensação dos tioésteres de p-cumaroil CoA com três unidades de malonil CoA, formando-se uma chalcona (74). A CHI catalisa a ciclização intramolecular da chalcona para formar uma flavanona, a (2S)-naringenina, precursor de muitos flavonóides incluindo a sub-família das antocianinas (75).

As reacções de oxidação envolvendo quer enzimas dependentes do ferro (com e sem grupos heme) e do 2-oxoglutarato desempenham um papel central na biossíntese de flavonóides. Várias enzimas pertencentes a este grupo, incluindo as enzimas F3H, FLS e LDOX têm uma sequência directamente relacionada e catalisam a oxidação do anel "C" do flavonóide (76).

A oxidação de (2S)-flavanonas a desidroflavonóis (3-OH-flavanonas) é catalisada pela enzima F3H (77). A enzima FLS catalisa a insaturação de (2R,3R)-*trans*-desidroflavonóis para formar os flavonóis (78).

A oxidação da (2R,3S,4S)-*cis*-leucoantocianidina catalisada pela enzima LDOX (ou antocianidina sintetase, ANS) dá origem a 3-OH-antocianidinas (79, 80).

As enzimas F3H e LDOX são membros da família de oxigenases sem grupos heme dependentes de ferro e de 2-oxo-glutarato, assim como a enzima FS1 que catalisa a insaturação da (2S)-flavanona, uma das vias biossíntéticas que leva à formação de flavonas (81, 82).

A flavonol 6-hidroxilase é outra oxigenase dependente do 2-oxoglutarato que catalisa a oxidação do anel A (83, 84).

A enzima DFR catalisa a redução de desidroflavonóis a flavan-3,4-dióis, que é o substrato para a biossíntese de antocianinas envolvendo a LDOX e 3-*O*-glicosiltransferases (UFGT; RT) (85). A DFR também está envolvida na formação de protoantocianidinas pela acção da LAR. Esta última enzima catalisa a síntese de

catequina, o monómero iniciador na formação de taninos condensados ou proantocianidinas a partir de 3,4-cis-leucocianidina, o primeiro passo dedicado à biossíntese de proantocianidinas (86).

A enzima IFR catalisa a redução esteroespecífica da 2'-hidroxiformononetina a (3R) isoflavanona (87). A enzima VR catalisa a redução da vestitona a 7,2'-di-hidroxi-4'-metoxi-isoflavanol (DMI), e a DMI desidratase (DMID) catalisa a formação do anel éter com perda de água para formar a fitoalexina medicarpina (88, 89).

A oxidação dos aneís A e B e a insaturação das flavanonas é catalisada por heme-oxigenases do citocromo P450. O padrão de hidroxilação do anel B depende do fenilpropanóide utilizado na biossíntese do flavonóide e de enzimas pertencentes ao citocromo P450. O anel B hidroxilado em 4' tem origem no *p*-cumaroil CoA utilizado pela enzima CHS na biossíntese das chalconas. Alternativamente, a molécula utilizada pode ser *o*-di-hidroxilo cafeoil CoA formando-se uma eriodictiolchalcona e flavonóis 3', 4' hidroxilados, embora esta via pareça ser pouco habitual. Regra geral a hidroxilação do anel B é determinada pela presença e actividade das enzimas F3'H (hidroxilação no carbono 3') e F3'5'H (hidroxilação nos carbonos 3' e 5'). Estas enzimas hidroxilam um vasta gama de flavonóides incluindo o desidrocampferol, o campferol e a apigenina (90).

A formação de esqueletos de isoflavonas é um processo crítico da biossíntese de isoflavonóides e ocorre em dois passos: o primeiro consiste na migração do grupo 1,2-arilo da flavanona formando-se a 2-hidroxi-isoflavanona que é catalisado pela enzima IFS. O segundo passo consiste na introdução da dupla ligação entre C-2 e C-3, e é catalisado por uma desidratase (91).

A hidroxilação das isoflavonas, catalisada pela enzima I2'H é muito importante na biossíntese das fitoalexinas biologicamente activas (92).

A síntese de flavonas é caracterizada por uma grande diversidade de mecanismos de reacção operando no mesmo substrato para obter o mesmo produto. Num desses mecanismos, a FS1 retira dois electrões de C-2 e C-3 da flavanona para produzir a flavona. Outro mecanismo possível depende de uma mono-oxigenase do citocromo P450, a enzima FS2, que catalisa a formação de uma hipotética 2-hidroxilflavanona, que é subsequentemente desidratada a flavona (93, 94).

2.2.7.5. Substituições mais comuns na estrutura dos flavonóides

Existem muitos flavonóides e isoflavonóides contendo grupos metoxilo. A metilação em locais específicos modela a actividade *in vivo*, limitando o número de hidroxilos reactivos, alterando as propriedades de solubilidade do produto resultante e

consequentemente determinando quando a molécula interage com os receptores celulares. As *O*-metiltransferases (OMT's) de moléculas de baixo peso molecular das plantas usam S-adenosil-L-metionina como fonte de metilo, obtendo-se S-adenosil-L-homocisteína e os éteres metilados. A isoflavona *O*-metiltransferase (IOMT) é essencial na biossíntese da medicarpina, uma das principais fitoalexinas (95).

O número de hidroxilos da estrutura base dos flavonóides pode ser aumentado por polifenol oxidases (PPO). A aureusidina sintetase (AS), uma enzima homóloga das PPO é muito importantes na biossíntese das auronas, os flavonóides responsáveis pela cor amarela brilhante de muitas plantas ornamentais. A AS catalisa duas transformações químicas: a hidroxilação e a ciclização oxidativa (2' α desidrogenação) das chalconas precursoras das auronas (96).

Os flavonóides podem existir na forma livre na natureza, mas a forma glicosídica é mais comum. A glicosilação torna os flavonóides menos reactivos e mais solúveis em água, podendo ser considerada uma forma de protecção nas plantas pois evita danos citoplasmáticos e permite o armazenamento nos vacúolos. A conjugação de açúcares com moléculas nucleofílicas reduz a possibilidade de transferência de electrões da genina para outros componentes celulares, diminuindo desta forma a sua reactividade e consequentemente aumentando a estabilidade da molécula. Uma vez que os locais nucleofílicos são em muitos casos a parte da molécula que interage de forma prejudicial com outros componentes celulares, a adição de açúcares bloqueia o local activo e consequentemente reduz a toxicidade (97).

A glicosilação ocorre em átomos de oxigénio (grupos OH e COOH), azoto, enxofre e carbono e é catalisada por glicosiltransferases (GT's) que usam açúcares activados com nucleótidos como substratos doadores. Nas plantas existe uma grande família multigene de GT's, conjugando metabolitos secundários, hormonas, toxinas ambientais bióticas e abióticas e com acção directa na homeostase celular. As GT's são régio e estéreo selectivas mas reconhecem substratos com características muito distintas. Pensa-se que na formação de heterósidos flavonóidicos as enzimas envolvidas são as UGT's (UDP glicosiltransferases) que utilizam unidades de açúcar activadas com UDP (98, 99). As moléculas dadoras típicas para as GT's são a UDP-glucose e a UDP-ácido glucorónico, sendo menos frequentes a UDP-ramnose, UDP-xilose e UDP-galactose (69). As moléculas aceitadoras mais comuns são compostos hidroxilados. A conjugação com açúcares constitui uma modificação de relevo, sendo muitas vezes o último passo da biossíntese. Uma vasta gama de açúcares pode ser conjugada independentemente (monoglicósidos), em paralelo ou em cadeia (di, triglicósidos, etc) (Figura 17), (97).

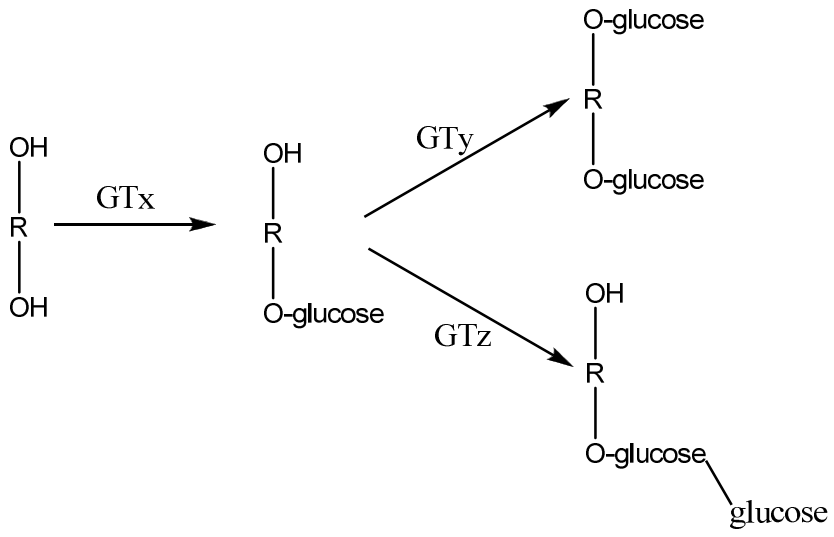


Figura 17. Formação de mono e di-glicósidos por glicosiltransferases [adaptada de (97)].
A enzima GTx catalisa a síntese de um monoglicósido. As enzimas GTy e GTz sintetisam duas formas de di-glucósidos. R: genina

Os açúcares ligados não têm um efeito significativo na absorção UV. Por esta razão estes compostos encontram-se muitas vezes localizados nas células da epiderme, conferindo protecção contra os raios ultra-violeta. Pensa-se que muitos heterósidos flavonóidicos podem ter uma função na interacção das plantas com os herbívoros associados e entre plantas e bactérias fixadoras de azoto (100, 101).

A hidrólise de heterósidos por β-glicosidases é uma parte importante e complementar do seu metabolismo.

Os heterósidos flavonóidicos encontram-se muitas vezes acilados com ácidos alifáticos e aromáticos, sobretudo acético, malónico, *p*-cumárico e ferúlico. A formação destes ésteres é o último passo da via biossintética, sendo catalisada por aciltransferases. Estas enzimas geralmente são pouco selectivas relativamente ao grupo acilo mas têm uma grande selectividade para o substrato a ser esterificado. As aciltransferases requerem como dador do grupo acilo a correspondente acil-coenzima A (102).

2.2.7.6. Compostos fenólicos em variedades *B. oleracea*

Os vegetais, e mais especificamente as variedades de couve da éspecie *B. oleracea,* são conhecidos por contribuírem para os compostos fenólicos ingeridos numa dieta normal. Nas variedades desta espécie previamente caracterizadas verificou-se que o seu perfil fenólico é constituído sobretudo por derivados do campferol e do ácido sinápico, como se pode ver na Tabela 4 e na Tabela 5. Das variedades mais consumidas em Portugal, nomeadamente os brócolos (inflorescências de *Brassica oleracea* L. var. *italica*), a couve-flor (*Brassica oleracea L.* var*. botrytis),* as couves de Bruxelas *(Brassica oleracea L.* var*. gemmifera*), o repolho (*Brassica oleracea* var*. capitata*) e as couves galega e lombarda (*Brassica oleracea* L. var. *acephala*) apenas os brócolos (103), os sub-produtos da couve-flor (104) e mais recentemente a couve Galega (105) e a couve lombarda (106) foram exaustivamente caracterizados.

A Tabela 4 contém alguns dos principais trabalhos que contribuíram para o esclarecimento do tipo de compostos fenólicos presentes em variedades de *B. Oleracea* (referidas pelo nome vernáculo). Estes compostos variam no tipo de glicosilações e acilações das 3 geninas mais comuns (o campferol, a quercetina e a isoramnetina) sendo necessário empregar técnicas de LC-MS^n para obter uma caracterização estrutural completa. Na Tabela 5 estão descritos os compostos fenólicos dos sub-produtos da couve-flor (23 compostos diferentes) e dos brócolos (40 compostos diferentes), ilustrando a complexidade do perfil fenólico desta espécie (103, 104).

Tabela 4. Compostos fenólicos identificados em variedades de couve da espécie *B. oleracea.*

Couve	Compostos identificados	Técnica	Ano	Refª
Identificação de alguns compostos				
Couve roxa	3 Antocianinas	UV	1969	(107)
Brócolos	3 Flavonóis	UV	1973	(108)
Couve roxa	5 antocianinas	MS; RMN	1987	(109)
Couve roxa	3 antocianinas	MS; RMN	1987	(110)
Repolho	7 flavonóis	RMN; GC-MS	1993	(111)
Brócolos	4 HCA	RMN	1997	(112)
Brócolos	5 flavonóis	RMN; MS	1998	(113)
Repolho	4 flavonóis	UV; RMN	1998	(101)
Tipo Galega	2 flavonóis	LC-MS	2003	(114)
Brócolos	2 CGA	LC-MS	2003	(115)
Caracterização do perfil fenólico				
Couve-Flor (sub-produtos)	19 flavonóis 4 HCA	$LC\text{-}MS^n$	2003	(104)
Brócolos	33 flavonóis 8 HCA	$LC\text{-}MS^n$	2004	(103)
Couve Roxa	18 antocianinas	$LC\text{-}MS^n$	2007	(116)
Couve Galega	15 flavonóis 4 HCA	$LC\text{-}MS^n$	2009	(105)
Lombarda	23 flavonóis 9 HCA	$LC\text{-}MS^n$	2009	(106)

Tabela 5. Caracterização em compostos fenólicos de extractos aquosos de brócolos (*Brassica oleracea* L. var. *itálica)* e de sub-produtos da couve-flor.

Brócolos	Couve-Flor (sub-produtos)
Querc-3-Caf-Softr-7-Glc	Querc-3-diGlc-7-Glc
Querc-3-Caf-Sof-7-Glc	Camp-3-triGlc-7-Glc
Querc-3-diCaf-Softr-7-Glc	Camp-3-Acil-diGlc-7-Glc
Camp-3-MeOCaf-Softr-7-Glc	Camp-3-diGlc-7-Glc
Camp-3-MeOCaf-Softr-7-Sof	Camp-3-Caf-diGlc 7-Glc
Camp-3-Caf-Sof-7-Glc	Camp-3-triGlc-7-diGlc
Camp-3-Caf-Softr-7-Sof	Camp 3-diGlc-7-diGlc
Querc-3-Sin-Softr-7-Glc	Querc-3-Sin-diGlc-7-Glc
Querc-3-Fer-Sof-7-Glc	Camp-3-Sin-diGlc-7-Glc
Querc-3-Fer-Softr-7-Glc	Camp-3-Fer-diGlc-7-Glc
Querc-3-pCum-Sof-7-Glc	Camp-3-Sin-triGlc-7-Glc
Querc-3-pCum-Softr-7-Glc	Camp-3-Sin-triGlc-7-diGlc
Camp-3-Sin-Softr-7-Sof	Camp-3-Fer-diGlc-7-diGlc
Camp-3-Sin-Softr-7-Glc	Camp-3-Acil-diGlc
Camp-3-Fer-Softr-7-Glc	Camp-3-Sin-triGlc
Camp-3-Fer-Softr-7-Sof	Camp-3-Sin-diGlc
Camp-3-Caf/pCum-Sof-7-Glc	Camp-3-diGlc
Camp-3-pCum-Softr-7-Sof	Camp-3-diSin-triGlc-7-diGlc
Camp-3,7-diGlc	Camp-3-diSin-triGlc-7-Glc
Isoram-3-Glc-7-Sof	1,2-diSin-diGlc
Querc-3-Sof	1-Sin-2-Fer-diGlc
Querc-3-Caf-Glc	1,2,2’-triSin-diGlc
Camp-3-Caf-Glc	1,2’-diSin-2-Fer-diGlc
Camp-3-Sof	
Querc-3-Caf/SinSoftr-7-Glc	
Camp-3-MeOCaf/Sin-Softr-7-Sof	
Camp-3-Caf/Sin-Softr-7-Sof	
Camp-3-Caf/Sin-Softr-7-Glc	
Querc-3-diSin-Softr-7-Glc	
Camp-3-diSin-Softr-7-Sof	
Querc-3-Fer/Sin-Softr-7-Glc	
Camp-3-diSin-Softr-7-Glc	
Camp-3-Fer/Sin-Softr-7-Sof	
1,2-diSin-Genc	
1-Sin-2-Fer-Genc	
1,2-diFer-Genc	
1,2,2’-trisin-Genc	
1,2’-diSin-2-Fer-Genc	
1-Sin-2,2’-diFer-Genc	
1,2,2’-triSin-Genc	
1,2,2’-triFer-Genc	

Caf: Cafeico; Cam: Campferol; Fer: Ferúlico; Genc: Genciobiose; Glc: Glucose; Isoram: Isoramnetina; MeOCaf: Metoxicafeico; pCum: Ácido *p*-cumárico; Querc: Quercetina; Sin: Sinápico; Sof: Soforósido; Softri: Soforotriósido.

2.3. Micropropagação de couve tronchuda

O termo micropropagação ou cultura *in vitro* define-se como a cultura em meios nutritivos e em condições estéreis de plantas, sementes, embriões, órgãos, tecidos, células ou protoplastos.

As culturas *in vitro* surgiram após o aparecimento da teoria da totipotência desenvolvida por Schwann e Schleiden, em 1838, segundo a qual cada uma das células da planta é autonómica e, em princípio, tem a capacidade de se regenerar para formar uma planta completa. Hänig, em 1904, isolou embriões (imaturos) de várias crucíferas tendo obtido plântulas viáveis.

As culturas *in vitro* tiveram um grande desenvolvimento nos anos 60 do século XX, após a descoberta dos reguladores de crescimento das plantas, tendo-se tornado um procedimento padrão na biotecnologia moderna. Actualmente, além da biotecnologia, esta técnica é amplamente utilizada em várias áreas, como a genética ou a fisiologia.

A cultura *in vitro* é um método de produção de biomassa utilizado com muito sucesso para uma grande variedade de espécies, que permite a produção de um grande número de plantas num tempo e espaço relativamente reduzidos. As plantas produzidas por este método, quando comparadas com plantas propagadas por sementes, geralmente apresentam maior vigor, qualidade uniforme e crescimento e maturação mais rápidos. Esta técnica é útil na clonagem de genótipos em grande escala.

Nas culturas *in vitro* o padrão normal de desenvolvimento da planta é quebrado. Um tecido isolado pode originar um “callus” ou tecido caloso (tecidos não organizados constituídos por células indiferenciadas e diferenciadas a dividir-se activamente) ou desenvolver-se de forma pouco habitual, formando órgãos por embriogenese somática. A possibilidade de crescimento de protoplastos (célula vegetal sem a parede celular) ou de células individuais permite efectuar manipulações de uma forma simplificada.

As culturas *in vitro* caracterizam-se por se desenvolverem em micro-escala com os factores ambientais, nutricionais e hormonais optimizados, na ausência de microrganismos (fungos, bactérias e vírus) e de outras pestes como os insectos e nemátodos.

O sucesso das culturas *in vitro* depende de vários factores, tais como o genótipo, idade, tipo de tecido, condições fisiológicas das plantas, meio de cultura, fitorreguladores e meio gasoso. Estes factores podem influenciar a produção dos metabolitos pretendidos e as taxas de multiplicação (117).

2.3.1. Reguladores de crescimento ou fitoreguladores

As hormonas são por definição compostos orgânicos naturalmente produzidos pelos organismos que influenciam o seu crescimento e desenvolvimento através de múltiplos mecanismos. A sua actividade é normalmente exercida num local diferente do local onde são produzidas, sendo necessárias pequenas quantidades para exercer a sua acção. O conhecimento das hormonas naturais levou ao desenvolvimento de compostos sintéticos com funções semelhantes. Ao conjunto das hormonas naturais e sintéticas chama-se reguladores. Os reguladores de crescimento das plantas, ou fito-reguladores, especialmente as auxinas e as citocininas são dos factores mais relevantes nos diferentes meios de cultura. Actuam em baixas concentrações e promovem, entre outros, a multiplicação e alongamento celulares (118).

2.3.1.1. Auxinas

As auxinas são substâncias produzidas nos ápices caulinares, nas raízes, nas sementes em germinação, nos meristemas de cicatrização, nas folhas novas e nos frutos. A principal auxina natural é o ácido indolacético (IAA), formado a partir do triptofano.

As auxinas servem, em primeiro lugar, para regular o crescimento celular e o alongamento do caule, podendo observar-se uma relação directa entre o crescimento e o aumento de concentração de IAA, até que se atinge uma concentração óptima. Concentrações acima desta provocam um crescimento mais reduzido, podendo mesmo ocorrer inibição do crescimento para concentrações muito elevadas de auxinas.

As auxinas também provocam a formação de tecido caloso (desdiferenciação), sendo a auxina sintética ácido 2,4 diclorofenoxiacético (2,4-D), a mais utilizada para este efeito (118).

2.3.1.2. Citocininas

Os citocininas são também factores de crecimento das plantas que possuem a capacidade de promover a divisão celular e a morfogénese e de provocar a mobilização de nutrientes, estando envolvidas na senescência foliar de muitos tecidos vegetais. Existem dois tipos de citocininas, as citocininas derivadas da adenina como a cinetina, zenatina e 6-benzilaminopurina e as citocininas derivadas da fenilureia como a difenilureia e o “thidiazuron” ou TDZ.

As citocininas, quando aplicadas em concentrações elevadas relativamente às auxinas, possuem a capacidade de promover a diferenciação celular em algumas células de tecido caloso, podendo afirmar-se que não possuem somente a função de divisão celular, mas também a de formação de órgãos (organogénese). Escolhendo a razão certa de citocininas/auxinas é possível promover a produção de uma planta completa a partir de tecido caloso: quando a concentração de citocinina é elevada relativamente à da auxina, formam-se as partes aéreas da planta; quando é baixa formam-se preferencialmente as raízes. As citocininas são geralmente utilizadas na concentração mais baixa que provoca a formação de rebentos e inibem o alongamento celular.

Nos casos de micropropagação, as citocininas provocam, muitas vezes, aumento da multiplicação com formação de rebentos. As citocininas mais utilizadas são a benziladenina (BAP) e a cinetina (118).

2.3.1.3. Giberelinas

As giberelinas são um grupo de compostos definido pela estrutura química relacionada e não pela sua actividade biológica. São factores de crescimento derivados de tetraciclinas diterpénicas, essenciais para o crescimento normal da planta, influenciando também vários processos biológicos.

As giberelinas são produzidas geralmente em tecidos jovens, meristemas, embriões e frutos não maduros, promovendo o alongamento celular e induzindo a floração.

O ácido giberélico tem a capacidade de influenciar o alongamento caulinar, interferindo na diferenciação das folhas, fotomorfogénese, desenvolvimento do pólen e florescimento, sendo fundamental no regulamento de vários aspectos do desenvolvimento vegetativo e reprodutivo (119, 120).

2.3.1.4. Etileno e inibidores de etileno

Normalmente as plantas exibem um aumento da produção de etileno quando sujeitas a stress ambiental ou ataque patogénico. A produção de etileno é uma das causas prováveis de envelhecimento precoce das plantas em cultura *in vitro.* Nas culturas de plantas do género *Brassica* é muito importante a existência de inibidores de etileno, como por exemplo o nitrato de prata (121). A utilização de ácido ascórbico pode também ser benéfica (122).

2.4. Larva de P. brassicae

A larva de *P. brassicae* L. (Lepidoptera: Pieridae) é uma praga frequente da couve tronchuda, sendo uma das principais causas de perdas nesta cultura. A frequência de infestação das culturas de couve tronchuda justifica a caracterização fitoquímica e avaliação do potencial antioxidante de extractos aquosos da larva de *P. brassicae*, com o objectivo de avaliar a sua utilização como fonte de compostos bioactivos potencialmente benéficos, e assim tirar partido das infestações nas culturas por esta praga.

As larvas de *Pieris brassicae* possuem elevada afinidade para crucíferas, tendo esta associação sido relacionada com a presença de glucosinolatos nas plantas (123). Além dos glucosinolatos, os flavonóides podem modular o comportamento alimentar das larvas, assim como a oviposição dos insectos adultos (124).

O sequestro de flavonóides é relativamente comum em insectos Lepidoptera, que os utilizam na pigmentação das asas. De uma forma geral, a captação de flavonóides pelos insectos aumenta as suas capacidades de sobrevivência (125). Como os insectos são incapazes de biossintetisar flavonóides, a presença destes tem sempre uma origem alimentar. A absorção e metabolismo dos flavonóides depende muito da composição específica da planta hospedeira. Sabe-se que as larvas podem discriminar entre os flavonóides presentes na planta, sequestrando apenas uma parte específica e que os flavonóides sequestrados são sujeitos a metabolização (126, 127).

Tal como nos mamíferos, os compostos fenólicos das plantas podem ter propriedades antioxidantes benéficas para os insectos herbívoros que os ingerem (128).

O lúmen intestinal de larvas de insectos Lepidoptera (como a larva de *Orgya leucostigma)*, que se alimentam de plantas ricas em compostos fenólicos, tem níveis elevados de ascorbato e GSH (129). Os compostos fenólicos podem ser anti ou pró-oxidantes dependendo das suas propriedades químicas e do ambiente físico-químico. O intestino das larvas de lepidoptera é muito alcalino, constituindo um ambiente químico particularmente apropriado para a oxidação de compostos fenólicos. Os insectos herbívoros desenvolveram um sistema defensivo baseado em condições químicas do lúmen intestinal que reduzem ou eliminam o comportamento potencialmente pró-oxidante dos compostos fenólicos ingeridos (129). As moléculas antioxidantes endógenas podem ter a função de destoxificação de compostos fenólicos oxidados ou das espécies reactivas geradas em ciclos redox em que estes

compostos participam. Por exemplo, verificou-se que o fluido mesenterial de larvas de *Eliothis virescens* (lagarta do tabaco) alimentada com folhas de tabaco (*Nicotiana tabacum*) tinha uma actividade antioxidante contra o radical 2,2'-azinobis-(3-etilbenzotiazoline-6-sulfonato (ABTS), superior ao das folhas maceradas. Além disso, verificou-se que a hemolinfa de larvas alimentadas com folhas geneticamente modificadas para produzirem maiores quantidades de fenóis tinham maior capacidade antioxidante do que a hemolinfa das larvas alimentadas com folhas normais, o que pode indicar que o aumento da actividade antioxidante se deve aos compostos fenólicos (128).

As interacções planta - hospedeiro são sistemas dinâmicos muito interessantes, em termos da variação dum metabolito individual ou de uma classe de compostos, podendo servir de base a estudos metabonómicos (130). A interacção das plantas com outros organismos, como as larvas de insectos herbívoros, pode resultar numa grande variação na resposta bioquímica das plantas (i.e cascatas de sinalização), conduzindo a uma situação mutuamente benéfica ou a uma defesa bioquímica contra o agressor.

2.5. Preparação das amostras de couve tronchuda

A preparação de amostras biológicas deve seguir procedimentos que garantam que não ocorrem alterações entre a colheita e os ensaios de caracterização química e de actividade biológica. Para isso, a actividade enzimática tem que ser rapidamente interrompida. A ultra-congelação em azoto líquido é muito eficaz, mas é necessário ter o cuidado de não descongelar parcialmente as amostras antes de extrair os metabolitos. A liofilizacão permite uma manipulação mais fácil das amostras e evita não só a actividade das enzimas como a dos transportadores (7).

Para proceder à caracterização química das amostras, as condições de extracção, como a temperatura, o tempo, e o solvente de extracção têm uma grande influência no tipo e quantidade de metabolitos extraídos (47). O processo de extracção deve ser um compromisso entre a recuperação de algumas classes de compostos e a minimização da decomposição de metabolitos mais sensíveis (7). Adicionalmente, quando se pretende estudar a actividade biológica de plantas utilizadas na alimentação humana, as condições de extracção devem mimetizar o modo de preparação habitual da planta.

2.5.1. Hidrólise de derivados de compostos fenólicos

As hidrólises químicas facilitam a identificação de compostos complexos, particularmente ao permitir a separação das geninas de açúcares e de grupos acilo. Para estudar os heterósidos flavonóidicos acilados pode proceder-se a 3 tipos de hidrólise diferentes:

Hidrólise ácida: na hidrólise ácida dos heterósidos flavonóidicos obtêm-se as geninas e os açúcares. As geninas podem ser posteriormente identificadas por LC-DAD ou LC-MS.

Hidrólise ácida suave: Em heterósidos flavonóidicos complexos, pode ser útil proceder à quebra parcial dos açúcares. Se a hidrólise ácida for realizada em condições controladas, podem eliminar-se os açúcares ligados ao hidroxilo no carbono 3, e analisar posteriormente os 7-*O*-heterósidos resultantes. Pode também conseguir-se a quebra de um açúcar terminal da uma cadeia glicosídica. Por exemplo, o campferol 3-*O*-soforósido-7-*O*-glucósido, quando submetido a uma hidrólise ácida suave origina o campferol 3,7-*O*-diglucósido. Neste tipo de hidrólise, usam-se tempos

e temperaturas de incubação mais baixos do que os necessários para a hidrólise de todos os açúcares (131).

Hidrólise alcalina: A hidrólise alcalina é tipicamente usada em compostos acilados. Após hidrólise alcalina de heterósidos flavonóidicos acilados com ácidos hidroxicinâmicos verifica-se o aparecimento dos ácidos hidroxicinâmicos e heterósidos flavonóidicos e o desaparecimento dos derivados acilados. Por vezes usa-se o extracto saponificado em LC-MS para identificar os heterósidos desacilados. Esta operação permite, por exemplo, distinguir os grupos substituintes glicosilo e cafeoílo que apresentam a mesma perda de massa (-162 u) (11).

2.6. Separação de compostos fenólicos por cromatografia líquida

A cromatografia líquida de fase reversa é particularmente útil para a análise de compostos polares ou moderamente polares e termolábeis como os polifenóis (132).

A separação baseia-se na diferença de afinidade dos compostos entre a fase estacionária e a fase móvel (133).

Na separação de flavonóides por LC utilizam-se colunas de fase reversa, habitualmente C_8 ou C_{18}. Para a análise de heterósidos flavonóidicos a coluna deve ser “end-capped”, isto é, com uma segunda funcionalização da sílica para reduzir os grupos silanol residuais, uma vez que estes grupos interagem com os açúcares, diminuindo a qualidade da separação (45).

A presença de compostos muito semelhantes nas amostras obriga à utilização de gradientes de eluentes com perfis mais complexos e maiores tempos de separação (132, 134). Habitualmente utiliza-se um gradiente com um sistema de eluentes binário, i. e; água acidificada e metanol ou acetonitrilo como modificador orgânico (134). A acidificação permite obter melhores resoluções, porque os grupos hidroxilo dos flavonóides são mantidos na sua forma acídica, aumentando assim o seu tempo de residência na coluna e diminuindo o alargamento dos picos, que é causado pela desprotonação. Os ácidos mais usados são o fórmico, o acético, e mais raramente o trifluoroacético, que são voláteis e por isso compatíveis com os sistemas de LC-MS (47). Destes, o ácido fórmico normalmente dá melhores resultados que o acético. Enquanto a adição do ácido fórmico só afecta a resolução, a adição de ácido acético também diminui consideravelmente o tempo de retenção, o que pode ser explicado pela sua menor capacidade de formar pares iónicos. Como consequência, utilizam-se muitas vezes menores concentrações de ácido acético na fase móvel para obter melhores resoluções. Além disso, em LC-MS a acidificação suprime a desprotonação e consequentemente diminui a eficiência de ionização no modo negativo, conseguindo-se melhores resultados se a acidificação for limitada. Um eluente adequado deve permitir a formação de iões em solução e uma nebulização e dessolvatação fáceis. Em termos de sensibilidade, o ácido acético dá melhores resultados do que o ácido fórmico.

Para a análise de ácidos clorogénicos por LC-MS é necessário recorrer aos modificadores de matriz (acetato ou formiato de amónio) para promover a formação de iões em solução na interface entre o LC e o MS (45, 135).

2.7. Identificação dos compostos fenólicos

A identificação de compostos fenólicos faz-se muitas vezes com detectores de díodos (DAD) tirando partido do espectro de ultra-violeta/vísivel (UV/vis) característico de cada sub-grupo de flavonóides e de ácidos fenólicos. Os espectros de UV/vis são muito úteis na identificação das geninas, mas para a identificação dos seus derivados presentes nas amostras, é necessário recorrer aos detectores de massa (MS). A combinação da cromatografia líquida como método de separação e dos detectores DAD e MS na identificação dos compostos é actualmente a técnica mais usada para obter o perfil fenólico de extractos de plantas. Os dois detectores podem fornecer informação "on-line" para cada pico individual no cromatograma, tornando possível a sua identificação por comparação com espectros de UV-vis e de massa de bibliotecas e por comparação com padrões (136).

Na análise de heterósidos, a ressonância magnética nuclear (NMR) é particularmente útil para a diferenciação de isómeros dos açúcares e dos padrões de substituição dos anéis aromáticos. Contudo, são necessárias técnicas de MS em série para obter informação sobre a massa molecular e grupos funcionais presentes (134).

2.7.1. Identificação dos compostos fenólicos pelo espectro UV/vis

Todos os polifenóis contêm pelo menos um anel aromático, e portanto absorvem luz ultra-violeta originando um espectro característico. A forma do espectro de UV-vis e a determinação rigorosa dos máximos de absorvância e inflexões são muito úteis na identificação destes compostos.

2.7.1.1. Ácidos hidroxicinâmicos

A maior parte dos ácidos hidroxicinâmicos absorve em duas regiões do espectro UV, apresentando um máximo entre 225-235 nm e outros dois máximos muito próximos, entre 290-330 nm. A dupla absorção nesta segunda região deve-se à presença dos isómeros *cis* e *trans*, dependendo a importância de cada máximo da quantidade de cada um dos isómeros (137). Quando o padrão de substituição nos ácidos cinâmicos é simétrico (p. ex ácido sinápico; ácido *p*-cumárico) só se verifica absorção na segunda região do espectro (48).

2.7.1.2. Flavonóides

Cada grupo de flavonóides origina um espectro de UV-vis com uma forma característica, permitindo com frequência uma identificação imediata (133). O LC com detecção por DAD é muito usado quando se pretende fazer identificação e quantificação por grupos de compostos flavonóidicos.

O espectro dos flavonóides caracteriza-se pela presença de dois máximos no seu espectro de UV-vis, um entre os 240-285 nm que se deve ao anel A (banda II), e outro que se encontra entre os 300-550 nm, que se deve ao padrão de substituição do anel B e conjugação do anel C (banda I). Nos flavonóis o máximo de absorção da banda I encontra-se entre os 328-385 nm (48).

A maior parte dos resíduos glicosilo e acilo são cromóforos fracos, não permitindo a identificação dos flavonóis seus derivados por LC-DAD (134). Contudo, os flavonóides acilados com ácidos hidroxicinâmicos (e.g. *p*-cumárico, cafeico e ferúlico) têm um espectro de UV característico que se assemelha à sobreposição dos espectros do flavonol com o ácido hidroxicinâmico, com um amplo máximo na zona dos 330 nm. A informação estrutural obtida destes espectros é menor do que a obtida dos espectros UV de flavonóides não acilados ou dos derivados de ácidos hidroxicinâmicos. Pode confirmar-se a presença de um flavonóide acilado fazendo uma hidrólise alcalina e verificando a presença no cromatograma da genina do flavonóide e do ácido hidroxicinâmico (103).

Os heterósidos de flavonóis apresentam um ligeiro desvio hipsocrómico na banda I. Por exemplo, o campferol com substituição no carbono 3 apresenta dois máximos a 267 e 349 nm e uma inflexão a 300 nm (11). Após hidrólise ácida, observa-se no espectro de UV-vis da genina um máximo na banda I a 367-371 nm.

Como os espectros de UV-vis dos heterósidos praticamente não apresentam diferenças, a diferenciação entre os diferentes heterósidos é feita com base no tempo de retenção (por exemplo os tetraglicósidos podem distinguir-se dos triglicósidos por tempos de retenção mais baixos) (101).

Os espectros de UV-vis são uma ferramenta complementar interessante na caracterização estrutural de flavonóides quando utilizados juntamente com técnicas espectroscópicas de MS e permitem uma identificação e quantificação simultânea.

2.7.2. Aplicação da espectrometria de massa à análise de compostos fenólicos

As técnicas de espectrometria de massa modernas ligadas às técnicas de separação por LC são actualmente muito utilizadas para a separação e identificação de polifenóis em extractos vegetais. Os detectores de massa são detectores universais com grande sensibilidade que podem fornecer informação da massa molecular e características estruturais de moléculas complexas. Além disso, as técnicas de LC e MS hifenadas permitem a análise directa dos extractos, obtendo-se bastante informação estrutural a partir de pequenas quantidades de amostra (47, 131).

Muitas vezes os espectros de MS não fornecem toda a informação necessária para estabelecer, sem ambiguidade, a estrutura de um composto desconhecido, sendo necessário recorrer a espectros de massa em série (Tandem MS/MS ou MS^n) para obter informação mais detalhada (47, 72). A espectrometria de massa em série compreende a selecção e isolamento de iões num intervalo apertado de massa/carga (ião precursor), a dissociação por colisão dos iões seleccionados ou isolados, e a análise de massa dos iões produto obtidos (138).

Os sistemas cromatografia líquida com detectores de massa são constituídos por 4 partes fundamentais, como se indica na Figura 18: o cromatógrafo, uma fonte de ionização dos compostos previamente separados, um detector de massa e um sistema de recolha de dados.

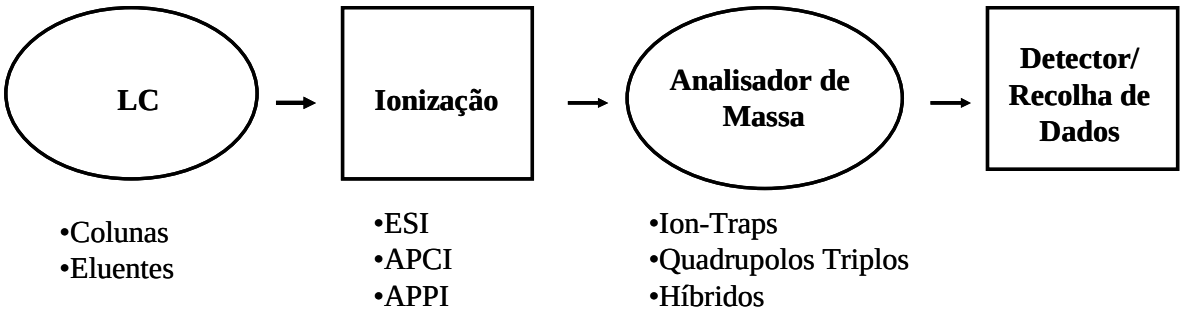


Figura 18. Esquema de um equipamento de LC-MS

Como a cromatografia líquida e os espectrometros de massa têm diferentes condições de operação é necessário utilizar interfaces na ligação destes equipamentos. A LC funciona com caudais de eluente elevados, pressões elevadas e

temperaturas da fase líquida próximas da temperatura ambiente; pelo contrário, a MS usa caudais baixos, vácuo elevado e fases gasosas com temperaturas elevadas (136).

A base da espectrometria de massa é a produção de iões que são separados ou filtrados de acordo com a sua razão massa (m) / carga (z) e depois detectados (136). A interface LC-MS deve permitir a ionização do analito e vaporização do eluato, remoção do excesso de vapor e extracção dos iões para o detector de massa.

Um dos traços característicos da MS reside na utilização de diferentes princípios físicos, quer para a ionização da amostra quer para a separação dos iões gerados (72). Os flavonóides são compostos polares, não voláteis e termicamente lábeis, o que dificultou a aplicação directa das primeiras técnicas de ionização usadas em MS, como o impacto electrónico (EI) ou a ionização química (CI). Estes métodos requerem que o analito esteja na fase gasosa para ser ionizado, o que obriga à derivatização dos grupos hidroxilo (metilação, trimetilsililação, e acetilação), sendo a informação estrutural obtida para os heterósidos derivatizados muito limitada (72). Mais recentemente surgiram os métodos de ionização à pressão atmosférica (API) particularmente adequados para os sistemas combinados de LC e MS. Nas técnicas de ionização à pressão atmosférica inclui-se a ionização por electrospray (ESI), a ionização química à pressão atmosférica (APCI), a foto-ionização à pressão atmosférica (APPI) e ainda combinações de dois tipos de fontes (ESI/APCI, APCI/APPI). A ionização por ESI é mais adequada para a análise de compostos térmicamente instáveis com maiores polaridades e pesos moleculares. Pelo contrário, a ionização por APCI ou por APPI é mais adequada para a análise de compostos de menor peso molecular e menor polaridade. Particularmente a ionização por ESI apresenta boa sensibilidade para a análise estrutural dos heterósidos flavonóidicos presentes nos extractos de plantas, sendo por isso uma das técnicas mais utilizadas (72, 132, 136).

A ionização por ESI consegue-se aplicando um potencial elevado a um capilar muito estreito por onde flui o eluente o que origina um nevoeiro de gotículas carregadas. Os iões são pré-formados na solução, ocorrendo depois a dessolvatação e fissão das gotículas, essencial para a formação de iões na fase gasosa. Os iões são depois filtrados para o analisador de massa (72, 136).

A ESI é considerada uma técnica de ionização suave, não produzindo muitos fragmentos, o que nem sempre permite uma boa elucidação da estrutura do composto (136). Esta técnica pode ser usada em modo ião negativo (NI) ou ião positivo (PI). O modo NI é bastante usado porque os iões fenolato formados são bastante estáveis, obtendo-se boas sensibilidades. No modo positivo, o ruído é relativamente alto, o que impede a detecção de alguns compostos. Comparativamente, o modo negativo é

menos afectado por sinais de impurezas interferentes ou ruído de fundo (47, 132). Além disso, os flavonóides originam um sinal baixo em modo positivo, provavelmente por não terem átomos de azoto, apresentam uma baixa basicidade na fase líquida, não se formando facilmente catiões.

A ionização por electrospray requer a utilização de caudais de eluente baixos (até 100 µl/min) para obter a formação de gotículas de tamanho reduzido. Isto obriga à partição (splitting) do caudal, permitindo a utilização simultânea de outros detectores como o UV-vis (72). A junção de espectros de UV-vis com os espectros de massa de primeira ordem obtidos com ESI fornece muita informação estrutural e permite a caracterização rápida de flavonóides, mesmo quando não existem compostos de referência ou estes são difíceis de obter (45).

Os iões formados na interface da LC podem ser analisados com espectrómetros de massa diferentes. Actualmente, os analisadores de massa mais utilizados em LC-ESI-MS são o “Ion trap”, o triplo quadrupolo e sistemas híbridos. Num instrumento com armadilha de iões (ion trap) seleccionam-se os iões gerados na fonte que são posteriormente dissociados por colisão induzida (collision induced decomposition, CID MS/MS) separados e detectados. Estes passos ocorrem sequencialmente no tempo, mas no mesmo espaço, sendo possível determinar as relações ião precursor-ião produto, o que não acontece com o triplo quadrupolo (132). A combinação da LC com ESI e Ion trap apresenta boa sensibilidade e especificidade e permite obter uma boa resolução de massas num amplo intervalo de massa (138).

2.7.3. Espectros de massa do campferol

Nas condições de LC-ESI-MS usadas na identificação de heterósidos flavonólicos o ião pseudo-molecular das geninas é virtualmente não dissociável. Contudo, a aplicação de energias de colisão superiores a 25 eV permite fragmentar geninas como o campferol e a quercetina e caracterizá-las estruturalmente (139).

Em modo positivo, a fragmentação do ião $[M+H]^+$ origina quebras simples do anel C que permitem a caracterização dos substituintes nos anéis A e B. Observam-se ainda iões formados em reacções de rearranjo acompanhadas de perdas de C_2H_2O, $CHO^{\cdot}$, CO e H_2O. As vias de fragmentação do campferol protonado estão esquematizadas na Figura 19.

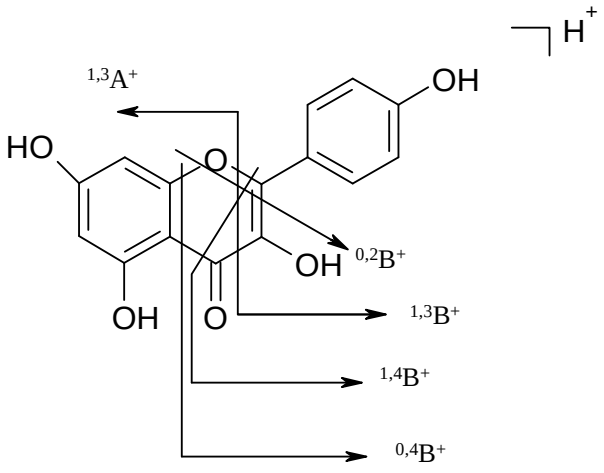


Figura 19. Estrutura do campferol protonado e principais fragmentações [adaptada de (139)].

Na Figura 19 os fragmentos $^{i,j}A^{+}$ e $^{i,j}B^{+}$ representam respectivamente os iões produto primários contendo os anéis A e B intactos, em que os expoentes i e j indicam as ligações do anel C que foram quebradas (139). Quando se usa o modo negativo os iões são marcados como $^{i,j}A^{-}$ e $^{i,j}B^{-}$, respectivamente (134). Os iões derivados de fragmentos pela perda de um fragmento X são marcados como [$^{i,j}A^{\pm}$ - X] e [$^{i,j}B^{\pm}$ - X].

Uma reacção importante na fragmentação de flavonóides é a reacção retro Diels-Alder (RDA), que pode ocorrer em estruturas cíclicas de 6 carbonos contendo uma ligação dupla e envolve a deslocalização de três pares de electrões (Figura 20). Obtêm-se dois fragmentos complementares $^{1,3}A^{+}$ e $^{1,3}B^{+}$, podendo a carga ficar retida em qualquer um deles. Os iões produto da quebra no anel C podem ser usados para determinar o número e natureza dos substituintes nos anéis A e B.

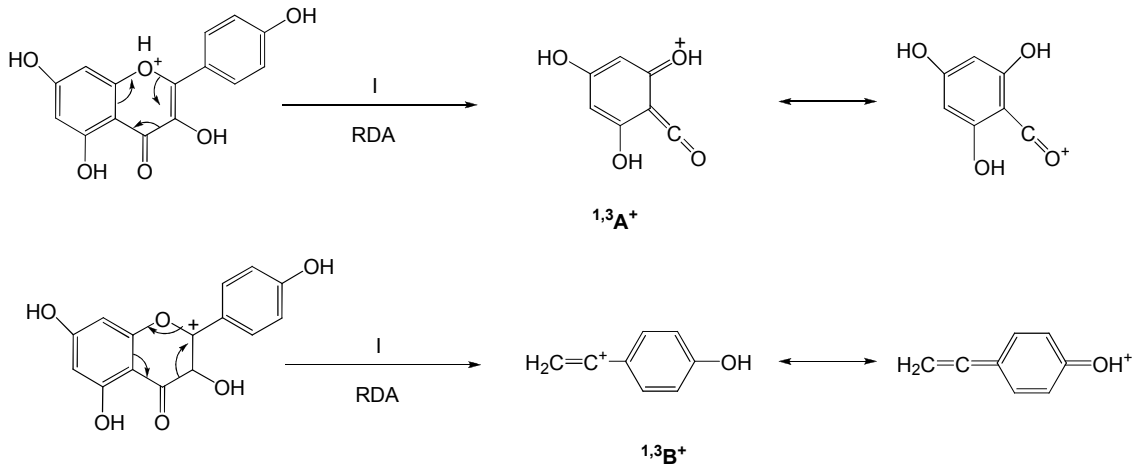


Figura 20. Fragmentação do campferol pela reacção de retro Diels-Alder (RDA).

As vias de fragmentação são quase independentes do modo de ionização (ESI ou APCI) e do tipo de instrumentos (triplo quadrupolo ou Ion Trap), mas podem haver diferenças significativas na abundância relativa de cada fragmento (134).

De uma forma geral, as fragmentações mais úteis para a identificação das geninas envolvem a quebra de duas ligações C – C do anel C nas posições 1/3, 0/2 e 0/4 (139, 140). Estas vias de fragmentação são designadas por I, II e III respectivamente, sendo que as vias I e III correspondem a fragmentações retro Diels-Alder.

No modo ião negativo, a fragmentação do ião pseudo-molecular do campferol ($[M-H]^-$) ocorre abruptamente, sendo obtidos mais de 70 fragmentos com baixa intensidade (138, 140). Adaptando o valor da energia de colisão podem obter-se iões formados em reacções de rearranjo do ião pseudo-molecular acompanhados de perdas de $OH^{\cdot}$, CO, CH_2O e C_2H_2O. Na Tabela 6, encontram-se os iões mais representativos do espectro de massa quando se usa este tipo de fragmentação, assim como a sua abundância relativa. As perdas sucessivas de CO e CO_2 envolvem o anel C. A perda de C_2H_2O envolve o anel A (141).

Tabela 6. Iões obtidos por ESI-MS/MS a partir do campferol desprotonado [adaptada de (141)].

Fragmento	Massa e	Abun	Perda de massa
$[M-H-CO]^-$	257 (3)		(-28)
$[M-H-C_2H_2O]^-$	243 (2)		(-42)
$[M-H-CO_2]^-$	241 (1)		(-44)
$[M-H-2CO]^-$	229 (3)		(-56)
$[M-H-CO_2-CO]^-$	213 (3)		(-72)
$[M-H-C_2H_2O-CO_2]^-$	199 (1)		(-86)
$^{1,3}A^-$	151 (1)		(-134)

2.7.4. Espectros de massa de heterósidos flavonólicos

A técnica de LC-ESI-MS^n permite identificar heterósidos flavonólicos muito complexos. Dada a sua relevância para a couve tronchuda, objecto de estudo deste trabalho, de seguida descrevem-se os padrões de fragmentação típicos de flavonóis glicosilados (com cadeias glicídicas contendo até 4 resíduos de açúcar) e de derivados de ácidos hidroxicinâmicos.

A partir dos espectros de massa pode saber-se a massa molecular do composto, a estrutura da genina (padrão de hidroxilação, ponto de ligação do anel B

ao anel C) e se existe metilação ou sulfatação do(s) hidroxilo(s) da genina. Relativamente à fracção glicosídica, é possível obter informação sobre o número de resíduos (mono, di, tri, tetrassacarídeos) e o tipo de açúcar (hexoses, desoxiesoses ou pentoses; ácidos glucurónicos). Também é possível obter informação sobre a esteroquímica do monossacarídeo terminal, a sequência da parte glicídica, o tipo de ligações interglicosídicas, a localização das ligações glicosídicas na genina e ainda informação acerca da acilação dos hidroxilos dos açúcares. Por outro lado, os espectros de MS não fornecem informação acerca da estereoquímica da ligação glicosídica nem permitem distinguir entre unidades de açúcar diastereoméricas (47, 72).

Nos espectro MS de heterósidos flavonólicos os principais iões são o ião pseudo-molecular $[M-H]^-$, um aducto do ácido fórmico $[M+45]^-$, e os iões correspondentes à perda de açúcar, por exemplo glucose $[M-Glu]^-$ ou $[M-2Glu]^-$ (132).

A activação por colisão de iões $[M-H]^-$ de heterósidos flavonóidicos leva à perda sequencial dos açúcares, permitindo determinar a sua ordem (138). A perda de todos os açúcares na fragmentação MS^n origina um espectro de massa semelhante ao das geninas livres (132).

A perda de açúcares em flavonóis *O*-glicosilados, com substituição característica das variedades de *B. oleracea* nos hidroxilos 3 e 7 ocorre tipicamente pela seguinte ordem:

1º Quebra dos açúcares não acilados no carbono 7
2º Quebra dos açúcares acilados
3º Quebra dos açúcares no carbono 3

Entre os heterósidos flavonólicos com o mesmo tipo de açúcar nos hidroxilos 3 e 7, a fragmentação ocorre preferencialmente no carbono 7, o que significa que nas condições usadas em MS a ligação glicosídica no carbono 7 é mais lábil que no carbono 3. A sensibilidade especial da ligação glicosídica no carbono 7 é também demonstrada pela habitual perda directa de resíduos diglucósido em vez da quebra sequencial de duas glucoses. Este comportamento em MS contrasta com a sensibilidade química destas ligações glicosídicas, uma vez que nas hidrólises ácidas suaves os açúcares no carbono 3 são sempre os primeiros a ser libertados, o que permite isolar e analisar por LC-MS os 7-glucósidos intermédios (104, 131).

A formação de geninas a partir de diglicósidos é mais favorecida do que quando apenas existe uma glicose, apesar da carga ficar na parte do flavonol e a força da ligação entre essa parte e a glucose simples ou dupla ser a mesma (132).

Nos compostos com dois ou mais açúcares, os iões resultantes da quebra das ligações glicosídicas entre os açúcares são geralmente pouco intensos (72). Em seguida são feitas algumas considerações sobre a nomenclatura usada para as fragmentações das cadeias glicídicas em MS (Figura 21).

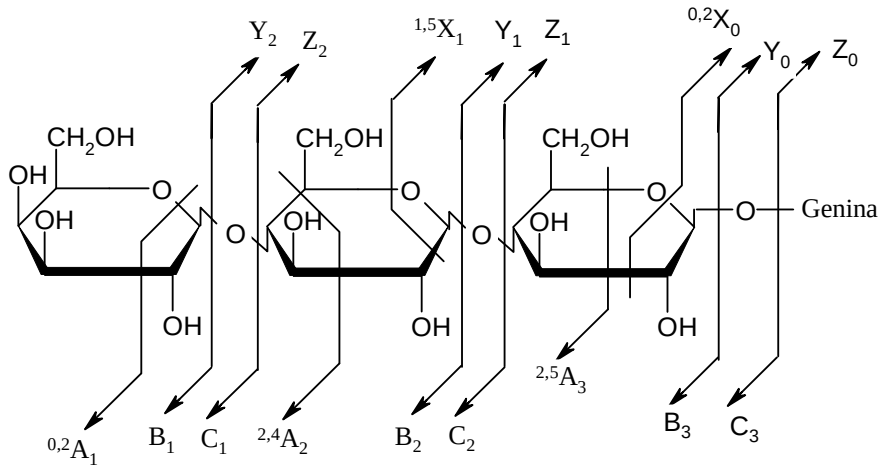


Figura 21. Nomenclatura usada na fragmentação de heterósidos flavonólicos [adaptada de (131)].

$^{K,1}X_j$, Y_j, Z_j - representam os iões contendo a genina. A letra (j), em índice, representa o número da ligação interglicosídica quebrada contando a partir da genina (ou a terminação redutora dos açucares), sendo a ligação glicosídica à genina numerada com zero. As letras em expoente (k e l) representam quebras dentro do anel dos açúcares.

A_i, B_i e C_i - designam os fragmentos glicosídicos em que i representa o número da ligação glícosidica quebrada.

Z e C – o oxigénio da ligação hemi-acetálica fica no fragmento do açúcar.

Y e B - o oxigénio da ligação hemi-acetálica (entre o aldeído e o fenol) fica no fragmento do flavonol.

^{K,l} X e ^{k,l}A - o fragmento do flavonol contem parte do anel do açúcar (ex: no fragmento $^{0,2}X_0$ a quebra ocorre nos carbonos 0 e 2 do açúcar ligado à genina).

As fragmentações mais comuns envolvem a quebra da ligação glicosídica, em que o átomo de oxigénio da ligação hemiacetálica é retido pelo ião que contem a genina, isto é, obtêm-se os fragmentos B_i e Y_j (142).

Os espectros de MS permitem a diferenciação entre os flavonóis glicosilados em que o açúcar se liga ao hidroxilo (*O*-heterósidos) e aqueles em que a ligação se dá directamente a um carbono do núcleo do flavonóide (*C*-heterósidos). A via de fragmentação dos flavonóis *O*-glicosilados envolve a quebra das ligações glícosídicas e eliminação dos açúcares, sendo a carga retida na genina ou no açúcar. Assim, nos

flavonoídes *O*-glicosilados observam-se sobretudo os fragmentos Y_j ou Z_j obtidos a partir do ião $[M-H]^-$. Nos *C*-glicósidos observa-se principalmente a fragmentação do açúcar (fragmentos X_j) (45, 72).

Em MS/MS produzem-se iões de primeira e de segunda geração. Na formação dos iões de primeira geração (A_i, X_j ou B_i, Y_j ou C_i, Z_j), está envolvida a quebra de uma só ligação glicosídica (ou no anel de açúcar). Os iões de segunda geração resultam da quebra de uma ligação glicosídica adicional a partir de um ião de primeira geração seleccionado (geralmente Y_j). As fragmentações de primeira e segunda geração podem levar à formação do mesmo tipo de produtos. Nos analisadores de massa com "Ion trap" é possível distinguir os 2 tipos de iões, uma vez que se pode estabelecer relações ião precursor - ião produto (142). As fragmentações de segunda geração e subsequentes são representadas por $Y^m{}_iY^n{}_j$, sendo que:

$Y^m{}_iY^n{}_j$ - Fragmento criado pela quebra de 2 açúcares substituintes em 2 posições diferentes da genina, em que m e n representam a posição do hidroxilo e i e j representam o número de glucósidos que ficam ligados (131). Se o ião for obtido em MS^n, a identificação começa no ião precursor de MS^{n-1} seguido do ião MS^n resultante. (Ex: o ião $Y^7{}_0Y^3{}_2$ obtido a partir do campferol 3-*O*-soforótriosido-7-*O*-soforósido denota a perda do açúcar terminal do triglicósido no carbono 3 ($Y^3{}_2$) a partir do fragmento $Y^7{}_0$ (perda total da glicosilação no carbono 7).

Durante a realização deste trabalho verificou-se que os heterósidos flavonólicos da couve tronchuda têm várias combinações de glicosilação nos hidroxilos 3 e 7: 3-glucósido, 3,7-diglucósido, 3-soforósido, 3-soforósido-7-glucósido, 3-glucósido-7-soforósido, 3-soforósido-7-soforósido, 3-soforotriósido-7-glucósido e 3-soforotriósido-7-soforósido e 7-soforósido-7-tetraglucósido (11).

Para os heterósidos livres e derivados acilados, os espectros de massa destes compostos podem ser adquiridos num intervalo m/z de 200 até 2000 e para os ácidos fenólicos e as geninas o intervalo m/z entre 90 e 400 é suficiente. Por exemplo, o ião pseudo-molecular $[M-H]^-$ do campferol-3-*O*-tetraglucósido-7-*O*-soforósido tem m/Z de 1257, enquanto que o ião desprotonado da genina $[Genina-2H/H]^-$, tem *m/z* 300 para a quercetina e *m/z* 285 para o campferol (103).

2.7.4.1. Heterósidos flavonólicos com duas hexoses

Os heterósidos flavonólicos contendo dois resíduos de hexose podem ser do tipo diglicósidos (X, Y), nos quais as unidades de açúcar se encontram ligadas a diferentes hidroxilos no núcleo do flavonol (habitualmente nos carbonos 3 e 7) ou podem existir

ligações interglucosídicas. Estas podem ser de vários tipos, mas nas variedades de *B. oleracea* as mais comuns são as soforoses [Glu(1→2)Glu] e genciobiose [Glu(1→6)Glu] (Figura 22), (103).

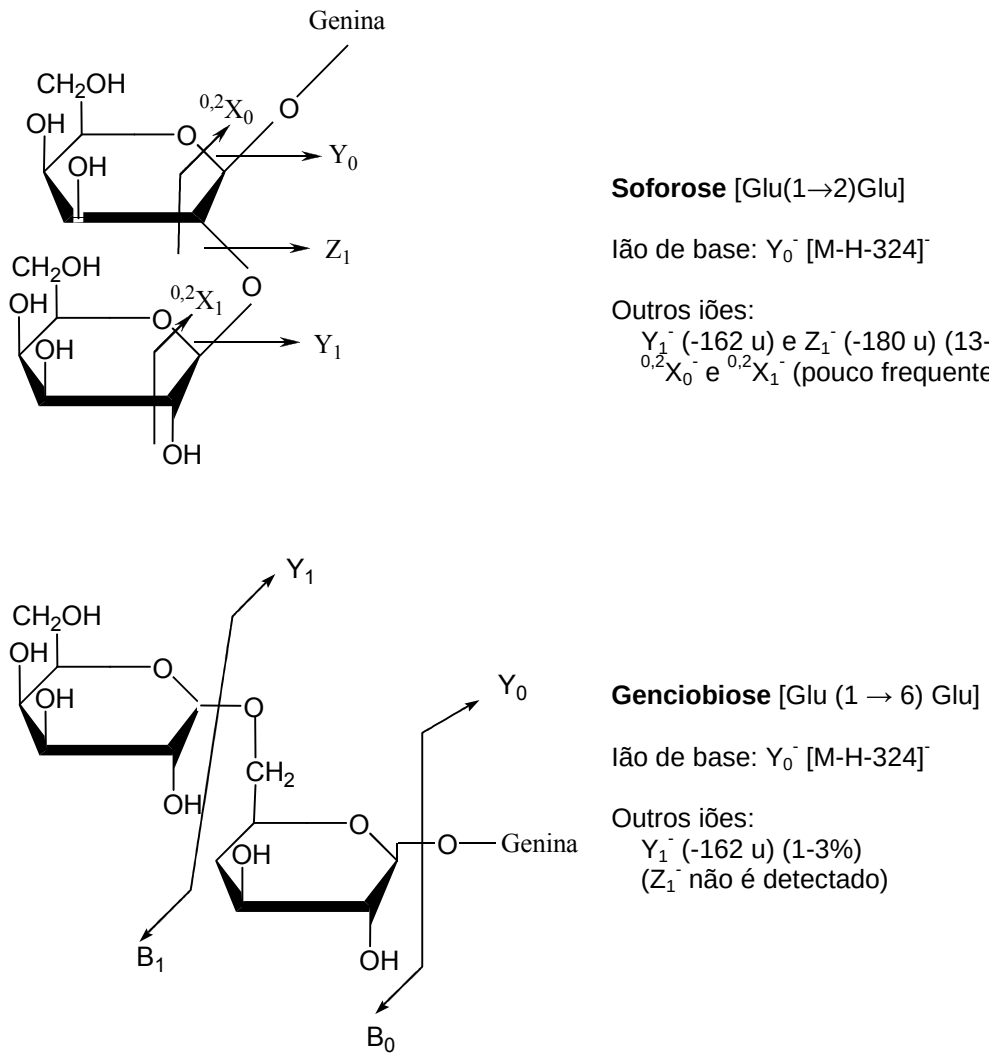


Figura 22. Padrão de fragmentação de soforose e de genciobiose.

A presença de genciobiósidos está associada aos derivados de ácidos hidroxicinâmicos, enquanto que os soforósidos são característicos dos heterósidos flavonólicos como foi previamente demonstrado por NMR de ^{1}H e ^{13}C (104). Estes três tipos de heterósidos podem ser distinguidos pela análise do seu espectro MS^2. Embora a soforose e a genciobiose apresentem o mesmo ião de base Y_0^- [M-H-324]$^-$, correspondente à perda de duas moléculas de glucose, na fragmentação da soforose, os iões intermédios Y_1^- e Z_1^-, formados pela quebra do açúcar terminal são relativamente abundantes (13-79%). Neste tipo de ligação interglucosídica os fragmentos $^{0,2}X_0^-$ e $^{0,2}X_1^-$ também podem ser detectados. Nos genciobiósidos, o ião Y_1^-

$[M-H-162]^-$ tem uma abundância relativa muito baixa (1-3%) e o ião Z_1^- $[M-H-180]^-$ não é detectado. Nos diglucósidos, o ião de base em MS^2 é formado pela perda de um glucosilo Y_1^- $[M-H-162]^-$, não se detectando o ião Z_1^- $[M-H-180]^-$, característico dos soforósidos. O ião Y_0^- $[M-H-324]^-$, que é o ião de base para os soforósidos e genciobiósidos tem uma abundância relativa de 30% nos heterósidos di-*O*-glucósidos.

2.7.4.2. Heterósidos flavonólicos com três hexoses

Nos heterósidos flavonólicos com 3 glucoses, o padrão mais comum de glicosilação é:

(a) Glicosilação no carbono 3 com soforotriose.

(b) Glicosilação no carbono 3 com soforose e no carbono 7 com glucose.

Os compostos com triglucosilação da genina em 3 hidroxilos situados em carbonos diferentes são menos comuns [nas sementes de couve tronchuda existe um composto em que o campferol tem glicoses ligadas aos carbonos 3, 7 e 4', (143)].

(**a**) Soforotriose: Aparecem vários iões com origem em fragmentações intermédias, característicos da ligação interglucosídica 1 → 2 (Figura 23)

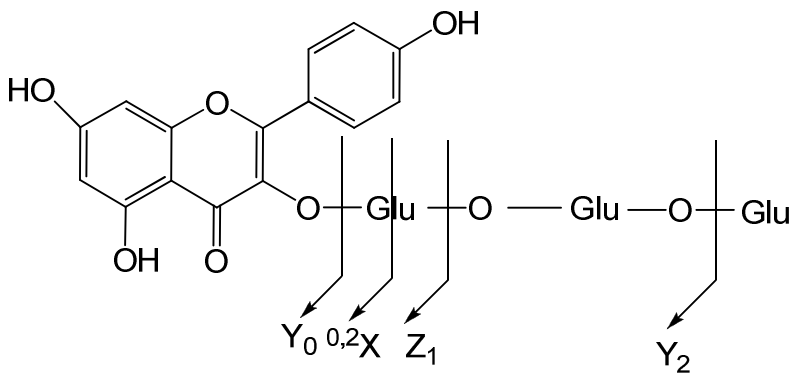


Figura 23. Fragmentação característica de soforotriósidos.

No espectro MS^2 do ião $[M-H]^-$ aparecem dois iões importantes; ${}^{0,2}X^-$ $[M-H-120]^-$ e Y_2^- $[M-H-162]^-$. Qualquer um destes fragmentos iónicos pode ser o pico de base. Outros fragmentos que aparecem com uma abundância relativa importante são os iões correspondentes à perda total dos açúcares Y_0^- ($[M-H-486]^-$) e o ião Z_1^- $[M-H-342]^-$.

Na análise MS^3 do fragmento Y_2^- (MS^3 [M-H → Y_2^-]), são obtidos os iões principais $Y_2Z_1^-$ (perda de 180 u) e $Y_2Y_0^-$ (correspondente à massa da genina em modo negativo).

(**b**) X-soforósido-Y-glucósido (Figura 24).

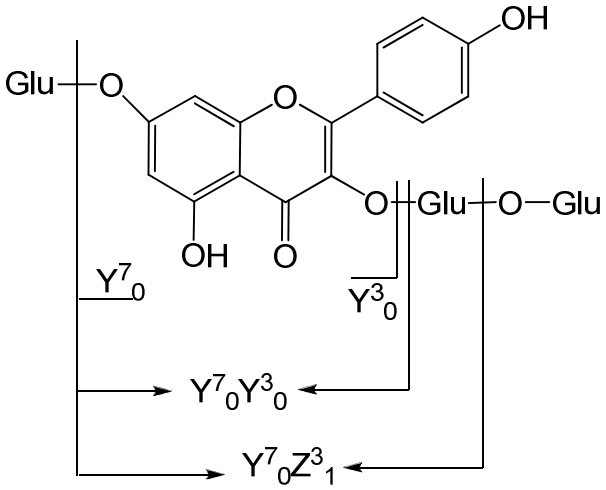


Figura 24. Fragmentação característica de X-soforósido-Y-glucósido.

Na análise MS^2 do ião $[M-H]^-$, detecta-se quase exclusivamente o ião $Y^7_0^-$ ($[M-H-162]^-$ resultante da quebra preferencial da glucose no carbono 7. Pode também observar-se o aparecimento de um pico minoritário $Y^3_0^-$ ($[M-H-324]^-$, com origem da perda de duas glucoses no carbono 3 do anel do flavonol.

A fragmentação do ião $Y^7_0^-$ em MS^3, é similar à observada para o ião Y_2^- no caso dos flavonóis soforotriósidos, obtendo-se os iões $Y^7_0^-Z^3_1^-$ e $Y^7_0^-Y^3_0^-$,em que o ião $Y^7_0^-Y^3_0^-$ corresponde à massa da genina em modo negativo e o ião $Y^7_0^-Z^3_1^-$ corresponde à perda da glicose terminal em $Y^7_0^-$ (-180 u).

De uma forma geral, os heterósidos flavonólicos com mais do que um açúcar ligados apenas a um hidroxilo podem distinguir-se daqueles em que os açúcares se ligam a mais do que um hidroxilo pela presença no seu espectro de massa MS^2 de iões do tipo $^{K,l}X$ e/ou Z_j relativamente abundantes. Nos compostos com açúcares ligados aos hidroxilos nos carbonos 3 e 7 observa-se o pico de base em MS^2 correspondente à perda total de glicosilação no carbono 7. A labilidade da ligação das soforoses ao hidroxilo no carbono 7 muitas vezes não permite observar em MS^2 os fragmentos característicos desta ligação. Mesmo assim, demonstrou-se esta ligação para os glicósidos no carbono 7 de alguns flavonóides (quercetina-3-*O*-glucósido-7-*O*-soforósido) através da análise MS^2 (103).

2.7.4.3. Heterósidos flavonólicos com quatro hexoses

Como exemplo, apresentam-se os padrões de fragmentação de flavonóis do tipo X-soforotriósido-Y-glucósido e X-soforósido-Y-soforósido. Em qualquer dos casos, nos espectros MS^2 de flavonóis com 4 açúcares, verifica-se que o pico de base indica a perda de todos os açúcares no carbono 7.

(**a**) X-soforotriósido-Y-glucósido (Figura 25).

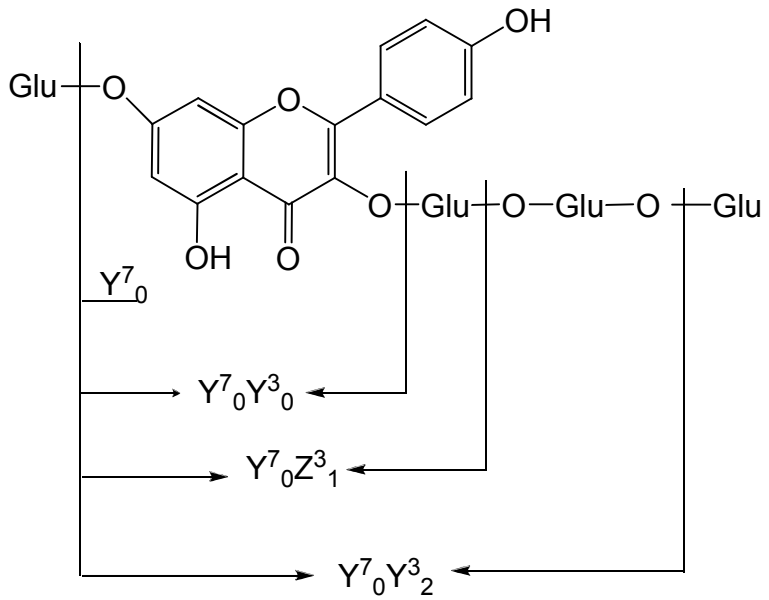


Figura 25. Fragmentação característica de X-soforotriósido-Y-glucósido.

A fragmentação de $[M-H]^-$ apenas origina o ião $Y^7_0{}^-$ ($[M-H-162]^-$, formado pela perda do radical glicosilo no carbono 7.

A fragmentação em MS^3 do ião $Y^7_0{}^-$ origina os iões $Y^7_0{}^-Y^3_2{}^-$ ($[Y^7_0 – 162]^-$), $Y^7_0{}^-Z^3_1{}^-$ ($[Y^7_0 – 342]^-$) e $Y^7_0{}^-Y^3_0{}^-$ ($[Y^7_0 – 486]^-$). Esta fragmentação é semelhante à observada para os fragmentos MS^2 do ião $[M-H]^-$ dos flavonóis soforotriósidos. Contudo, o ião $[M-H-120]^-$ muito abundante e característico para os flavonóis soforotriósidos não se detecta nos compostos com glicolisação 3-*O*-soforotriósido-7-*O*-glucósido.

(**b**) X-soforósido-Y-soforósido (Figura 26).

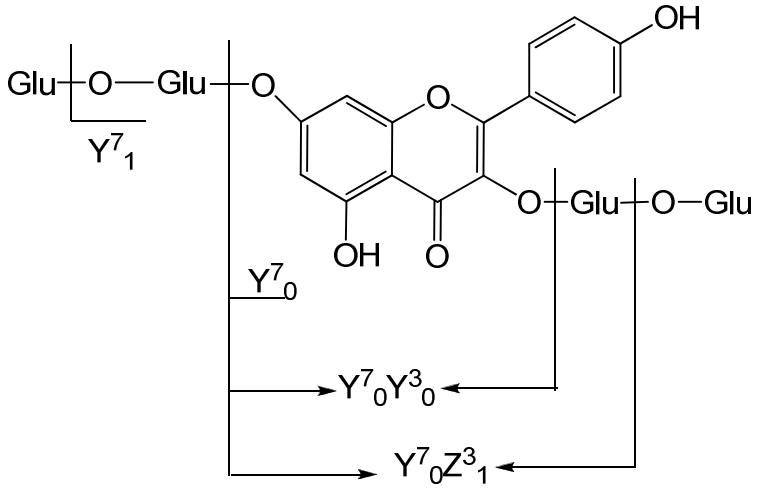


Figura 26. Fragmentação característica de X-soforósido-Y-soforósido.

Após a primeira fragmentação do ião $[M-H]^-$ do flavonol campferol 3-*O*-soforósido-7-*O*-soforósido detectam-se os iões $Y^7_1{}^-$ ($[M-H-162]^-$ e $Y^7_0{}^-$ ($[M-H-324]^-$, correspondendo à perda preferencial de um e dois açúcares no carbono 7, respectivamente. Contudo, verifica-se que a abundância relativa do ião $Y^7_1{}^-$ nunca ultrapassa os 7%.

A fragmentação do ião $Y^7_0{}^-$ em MS^3 origina os iões $Y^7_0{}^-Z^3_1{}^-$ ($[Y^7_0 – 180]^-$) e $Y^7_0{}^-Y^3_0{}^-$ ($[Y^7_0 – 324]^-$) produzidos como consequência da perda de um e dois açúcares no carbono 3 do anel.

2.7.4.4. Heterósidos flavonólicos com 5 hexoses

Nos flavonóis com um elevado grau de glicosilação como os tetraglicósidos e os pentaglicósidos a fragmentação parcial de duas ou três unidades de açúcar para formar iões intermédios em MS^2 é muito difícil de conseguir. Os iões que caracterizam a ligação 1→2 dos soforósidos e dos soforotriósidos têm uma abundância relativa muito baixa em MS^2, sendo contudo claramente detectados em MS^3. Por esta razão, a caracterização da ligação 1→2 é mais fácil e inequívoca na fragmentação MS^3 dos iões $Y^3_0{}^-$ e $Y^7_0{}^-$ obtidos a partir do ião $[M-H]^-$.

O composto usado como padrão para estudar os espectros de massa de flavonóides com cinco açúcares foi o campferol 3-*O*-soforotriósido-7-*O*-soforósido (Figura 27).

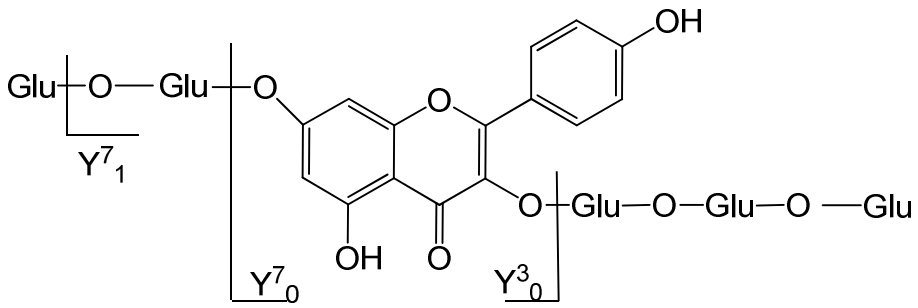


Figura 27. Fragmentação característica de 3-*O*-soforotriósido-7-*O*-soforósido.

Na análise MS^2 do ião $[M-H]^-$ verifica-se a presença de 3 picos significativos. Os iões $Y^7_1{}^-$ e $Y^7_0{}^-$ (pico de base) derivam da quebra principal do soforósido no carbono 7. A presença do pico $Y^7_1{}^-$ é característica da ligação interglicosídica 1→ 2, indicando a presença de uma unidade soforósido no carbono 7. Observa-se ainda a presença do terceiro ião $Y^3_0{}^-$ ($[M-H-486]^-$), com origem na perda de três glucoses no carbono 3.

A fragmentação em MS^3 do ião $Y^7_0{}^-$ dá origem a 3 iões característicos dos flavonóis soforotriósidos: $Y^7_0{}^-Y^3_2{}^-$ ($[Y^7_0 – 162]^-$), $Y^7_0{}^-Z^3_1{}^-$ ($[Y^7_0 – 342]^-$) e $Y^7_0{}^-Y^3_0{}^-$ ($[Y^7_0 – 486]^-$). A fragmentação em MS^3 do ião $Y^3_0{}^-$ também origina os três fragmentos característicos da quebra de flavonóis soforósidos: $Y^3_0{}^-Y^7_1{}^-$ ($[Y^3_0 – 162]^-$), $Y^3_0{}^-Z^7_1{}^-$ ($[Y^3_0 – 180]^-$) e $Y^3_0{}^-Y^7_0{}^-$ ($[Y^3_0 – 324]^-$).

2.7.4.5. Heterósidos flavonólicos com 6 hexoses

Apesar dos heterósidos com mais de 3 unidades de açúcar não serem frequentes na natureza, o aparecimento de tetra e penta glucósidos noutras variedades de *B. oleracea* (103, 104) levou à pesquisa de flavonóis glicosilados com 6 hexoses na couve tronchuda. O aparecimento de um composto com m/z 1257, coincidente com o valor do campferol hexaglucosilo desprotonado, com o padrão de fragmentação a seguir indicado, confirma a presença do campferol-3-*O*-tetraglucósido-7-*O*-soforósido em quantidades vestigiais na folha externa da couve tronchuda (11).

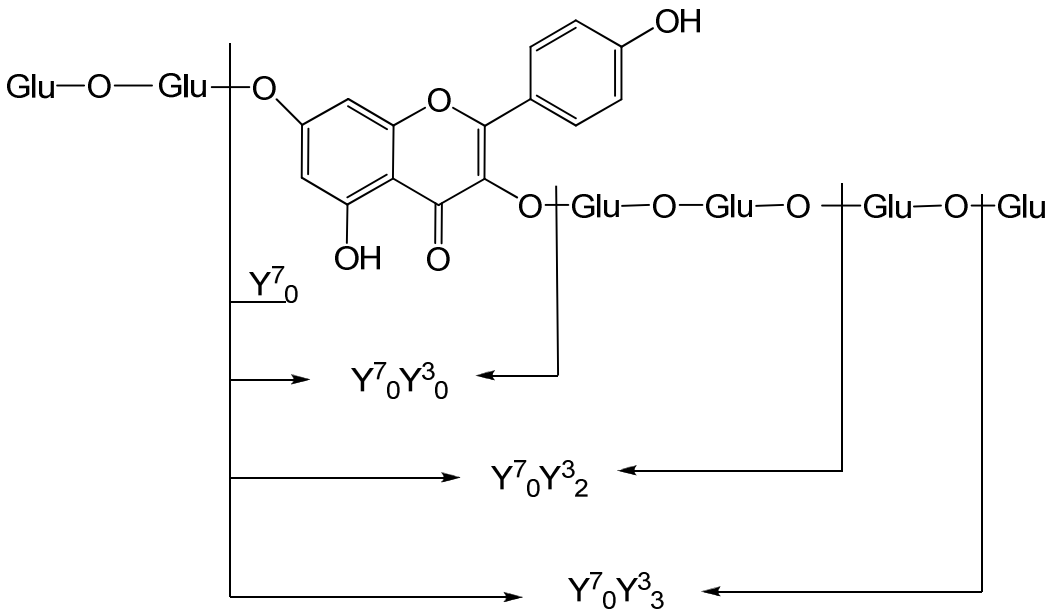


Figura 28. Fragmentação característica de 3-*O*-tetraglucósido-7-*O*-soforósido.

A fragmentação em MS^2 do ião $[M-H]^-$ leva à perda de 324 u, correspondente a duas unidades de glucose originando um ião $Y^7_0{}^-$ com m/z = 933 (pico de base); indicando a presença de um soforósido ligado a um hidroxilo no carbono 7 do campferol.

A fragmentação MS^3 do ião $Y^7_0{}^-$ origina 3 fragmentos: $Y^7_0{}^-Y^3_0{}^-$ correspondente ao ião da genina (m/z = 285); $Y^7_0{}^-Y^3_2{}^-$ com m/z = 609, correspondente à perda de 2 glucoses e $Y^7_0{}^-Y^3_3{}^-$ com m/z = 771, correspondente à perda de uma glucose, permitindo concluir que os restantes quatro açúcares deverão constituir um tetrassacarídeo ligado ao hidroxilo no carbono 3 do campferol (Figura 28).

2.7.5. Espectros de massa de heterósidos flavonólicos acilados

Nas variedades de *B. oleracea* são muito frequentes os heterósidos acilados de campferol ou quercetina, em que um ou mais açúcares se encontram esterificados com ácidos cinâmicos, nomeadamente com os ácidos *p*-cumárico, cafeico, sinápico, ferúlico e metoxicafeico.

A presença de compostos acilados num extracto pode ser confirmada comparando os cromatogramas do extracto original com o extracto saponificado.

Nos flavonóis acilados com substituições heterosídicas nos hidroxilos dos carbonos 3 e 7, verifica-se que a perda de substituintes ocorre habitualmente pela seguinte ordem:

1º Perda de glicosilação no carbono 7 em $MS^2[M-H]^-$,

2º Perda sequencial dos ácidos cinâmicos a partir do ião $Y^7_0{}^-$,

3º Perda da glicosilação no carbono 3 dos fragmentos desacilados.

confirmando que os açúcares acilados se encontram no carbono 3.

2.7.5.1. Compostos monoacilados

Nos compostos sem glicosilação no hidroxilo no carbono 7, o pico de base em MS^2 corresponde à perda do ácido cinâmico, não sendo detectados outros fragmentos (11, 144, 145).

Nos compostos glicosilados no hidroxilo no carbono 7, o pico de base em MS^2 corresponde quase sempre à perda deste açúcar, formando o ião $Y^7_0{}^-$, podendo ainda observar-se os fragmentos correspondentes à perda adicional do ácido cinâmico com intensidade relativa variável, dependendo do tipo de ácido (135, 145).

Na fragmentação em MS^3 do ião $Y^7_0{}^-$ observa-se o pico de base correspondente à perda do ácido cinâmico (135), sendo necessário uma fragmentação MS^4 para que ocorra a perda de glicosilação no carbono 3 formando-se o pico de base correspondente à massa da genina. Em MS^4 pode também observar-se a presença de outros fragmentos.

Os compostos monoacilados com glicosilação em três hidroxilos fenólicos diferentes (campferol-3,7-*O*-diglucosido-4'-*O*-(sinapoil)glucosido) apresentam um padrão de fragmentação característico. A fragmentação MS^n consiste em duas perdas sequenciais (MS^2 e MS^3) do radical glucosilo. Em MS^4 verifica-se a perda simultânea dos radicais glucosilo e sinapoilo originando o ião da genina (143).

2.7.5.2. Compostos diacilados

Nos compostos diacilados, tal como nos compostos monoacilados, no evento $MS^2[M-H]^-$ dá-se a perda de glicosilação no hidroxilo do carbono 7 formando-se o ião $Y^7_0{}^-$ (quase sempre o pico de base). Podem ainda observar-se os fragmentos correspondentes à perda adicional de um ácido cinâmico (Figura 29). Nos compostos diacilados com dois ácidos cinâmicos diferentes, observam-se por vezes dois picos correspondentes à perda de cada um dos ácidos, podendo estes dois fragmentos ter intensidades relativas muito diferentes (11, 145).

Na fragmentação em MS^3 do ião $Y^7_0{}^-$, observam-se os dois picos correspondentes à perda de um ácido cinâmico, em que um deles é o pico de base. Pode ainda observar-se um pico correspondente à perda simultânea dos dois ácidos, mas com uma intensidade relativa baixa.

No evento MS^4 do ião $[Y^7_0{}^-\text{-acilo}]^-$, ocorre a perda do segundo ácido cinâmico, sendo necessário um evento MS^5 para que haja a perda da glicosilação no carbono 3, formando-se o pico de base corresponde á genina (144).

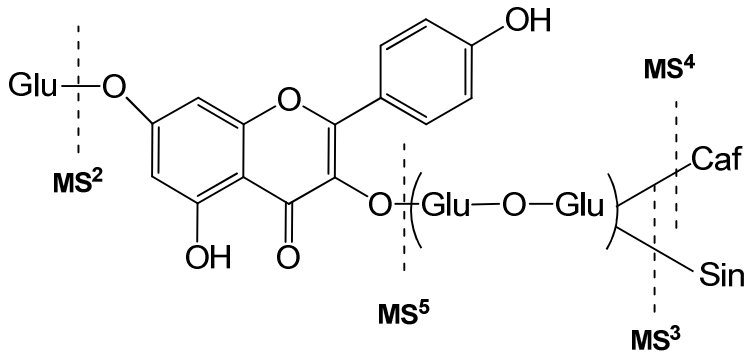


Figura 29. Fragmentação característica de heterósidos diacilados.

2.7.6. Espectros de massa de ácidos clorogénicos

Os ácidos clorogénicos (CGA) são uma família de ésteres formados entre alguns ácidos *trans* cinâmicos e o ácido quínico característicos dos grãos do café, mas que já foram encontrados noutras espécies, nomeadamente em variedades de *B. oleracea*, incluindo a couve tronchuda (135, 146, 147).

Em LC-ESI-MS os ácidos clorogénicos monoacilados originam o ião precursor $[CGA - H^+]^-$, o que permite saber de qual ácido cinâmico (cafeico, ferúlico ou *p*-cumárico) deriva o ácido clorogénico (147). A fragmentação em MS^2 é característica para cada um dos três possíveis isómeros (ligação do ácido hidroxicinâmico ao hidroxilo do carbono 3, 4 ou 5 do ácido quínico), permitindo identificar totalmente o composto pelos fragmentos obtidos e pela sua intensidade relativa. Os fragmentos obtidos são derivados do ácido quínico, do ácido quínico desidradato e do ácido hidroxicinâmico). A Tabela 7 contém dados de espectros de massa que permitiram identificar os CGA encontrados na couve tronchuda.

Tabela 7. m/z e intensidade relativa características dos fragmentos de ácidos clorogénicos analisados por espectrometria de massa [adaptada de (145)].

CGA	$[M-H]^-$	-$MS^2[M-H]^-$ $[Cinamoil-H]^-$	$[Quínico-H]^-$	$[Quínico-H_2O]^-$
Monoacilados				
3-*p*-CoQA	337	163 (100)	191 (6)	
4-*p*-CoQA	337			173 (100)
3-CQA	353	179 (39)	191 (100)	
4-CQA	353	179 (70)	191 (42)	173 (100)
3-FQA	367	193 (100)		
Diacilados		$[FQA-H]^-$		
CFQA	529	367 (60)	191 (100)	

CGA: ácido clorogénico; 3-*p*CoQA: ácido 3-*p*-cumaroilquínico; 4-*p*CoQA: ácido 4-*p*-cumaroilquinico; 3-CQA: ácido 3-cafeoilquínico; 4-CQA: ácido 4-cafeoilquínico; 3-FQA: ácido 3-feruloilquínico; CFQA: ácido cafeoilferuloilquínico.

2.7.7. Espectros de massa de heterósidos de ácidos hidroxicinâmicos

Os ésteres de ácidos cinâmicos e genciobiose são muito frequentes em variedades de *B. oleracea* (135). Na fragmentação MS o pico de base corresponde ao ião pseudo-molecular $[M-H]^-$. Nos seus espectros MS^n observam-se fragmentos característicos da perda do ácido cinâmico acompanhados ou não da perda de uma molécula de água e também os iões pseudo-moleculares do ácido hidroxicinâmico desprotonado (n depende de se tratar de um ácido monoacilado; diacilado ou triacilado). Os diferentes isómeros apresentam diferentes intensidades relativas para cada um dos iões, e nem todos os fragmentos aparecem para todos os isómeros.

Em MS^n encontram-se derivados acilados de genciobiose em que o padrão de fragmentação não corresponde ao dos ésteres de ácidos hidroxicinâmicos e genciobiose. Nestes compostos, o ião formado pela perda de um ácido hidroxicinâmico em $MS^2[M-H]^-$ não é abundante e a perda da fracção glicosídica não é completa, formando-se o ião $[^{0,2}X-H_2O]^-$. O ião $[M-H-H_2O]^-$ é abundante sendo o pico de base dos sinapoilgenciobiósidos. Comparando com a perda total do radical glicosilo, habitualmente encontrado nos compostos com ligação éster entre a genina e o açúcar, pensa-se que nos compostos com perda parcial do açúcar, a ligação entre as duas moléculas tem uma natureza diferente da ligação éster (143).

Em LC-ESI-MS^n nem sempre é possível determinar a posição de ligação do resíduo de ácido hidroxicinâmico ao açúcar (111).

2.7.7.1. Compostos monoacilados

Os fragmentos característicos em $MS^2[M-H]^-$ dos ésteres do ácido cinâmico são:

- Ião correspondente à perda de uma molécula de água $[M-H-H_2O]^-$
- Ião pseudo-molecular do ácido cinâmico desprotonado: [acido cinâmico - H]$^-$
- Ião correspondente ao ácido cinâmico desprotonado com perda de uma molécula de água: [ácido cinâmico -H-H_2O]$^-$

Podem também encontrar-se fragmentos correspondentes à quebra parcial do açúcar.

2.7.7.2. Compostos diacilados

Na couve tronchuda, nos derivados diacilados de ácidos hidroxicinâmicos, regra geral, pelo menos um dos ácidos é o sinápico, podendo o outro ser sinápico, cafeico, ferúlico ou metoxicafeico. Em MS^2 aparecem fragmentos correspondentes à quebra de um ácido. Se os dois ácidos cinâmicos forem diferentes, podem aparecer fragmentos com razão m/z correspondente à quebra de cada um deles. As intensidades destes dois fragmentos podem ser muito diferentes.

Os fragmentos característicos em $MS^2[M-H]^-$ dos ésteres do ácido cinâmico são:

- Perda de um grupo acilo (podem aparecer um ou dois iões diferentes se a genciobiose for acilada com dois ácidos cinâmicos diferentes)
- Iões correspondentes ao ácido cinâmico desprotonado [acido cinâmico - H]$^-$,
- Iões correspondentes aos ácidos cinâmicos desprotonados com perda de uma molécula de água [ácido cinâmico -H- H_2O]$^-$.

Os fragmentos característicos em $MS^3[(M-H)\rightarrow (M-H-Acilo)]^-$ são:

- [acido cinâmico - H]$^-$
- [ácido cinâmico -H-H_2O]$^-$

2.7.7.3. Compostos triacilados

Nos derivados triacilados de ácidos hidroxicinâmicos distinguiram-se 2 grupos de compostos na couve tronchuda: compostos contendo dois (ou três) resíduos de

ácido sinápico e compostos contendo 1 resíduo de ácido sinápico. Nos compostos triacilados com 2 ou 3 resíduos de ácido sinápico os fragmentos característicos em MS2[M-H]− são:

- Ião correspondente à perda de um grupo sinapoílo $[M\text{-acilo}]^-$
- Ião correspondente à perda de dois grupos sinapoílo, acompanhados da perda de uma molécula de água $[M\text{-acilo-acilo-}H_2O]^-$

Seleccionando o fragmento correspondente à perda de um grupo acilo, obtêm-se os fragmentos característicos em $MS^3[(M\text{-}H)\rightarrow (M\text{-}H\text{-Acilo})]^-$:

- Perda adicional de um grupo acilo: $[M\text{ -acilo-acilo}]^-$
- Perda adicional de um grupo acilo acompanhado da perda de uma molécula de água $[M\text{ -acilo-acilo-}H_2O]^-$
- $[\text{acido cinâmico - H}]^-$

No caso dos compostos triacilados com um só ácido sinápico, o espectro MS^2 pode apresentar mais do que um ião correspondente à perda de um grupo acilo. Podem ainda aparecer iões correspondentes à perda simultânea de 2 grupos acilo.

Os fragmentos característicos dos heterósidos não flavonólicos de ácidos hidroxicinâmicos encontram-se na Tabela 8.

2.7.8. Espectro de massa da sinapoilcolina

Foi ainda determinado outro composto característico das sementes no género *Brassica,* a sinapoilcolina ou sinapina, que apresenta um máximo de absorção no UV-vis a 325 nm e com os fragmentos iónicos em MS de modo positivo:

- MS: 310, (ião pseudo-molecular da sinapoilcolina $[M]^+$)
- $MS^2[M]^+$: 251 (ião correspondente à perda de colina $[M–(CH_3)_3N]^+$).

Tabela 8. Quebras de massa de derivados acilados de genciobiose ou glucose.

Monoacilados	$[M-H]^-$	$MS^2[M-H]^-$ $[M-H-H_2O]^-$	$[^{0,2}X-H_2O]^-$	$[Cinâmico-H]^-$	$[Cinâmico-H-H_2O]^-$
Sinapoil**g**luc	385	367	247	223	205
Sinapoil**g**enc	547	529	247	223	

Diacilados	$[M-H]^-$	$MS^2[M-H]^-$ $[M-Acilo]^-$	$[Cin-H]^-$	$[Cin-H-H_2O]$	$MS^3[(M-H)\rightarrow(M-H-Cin)]^-$ $[Cin-H]^-$	$[Cin-H-H_2O]$
*di***S**inapoil**g**luc	591	367	223	205		205
*di***S**inapoil**g**enc	753	529	223		223	205
Sinapoil**F**eruloil**g**enc	723	529 (-Fer) 499 (-Sin)			193	175
Sinapoil**M**etoxi**C**afeoil**g**enc	739	515 (-Sin)				191

Triacilados

(2 ou 3 sinápicos)	$[M-H]^-$	$MS^2[M-H]^-$ $[M-acilo]^-$	$[M-Acilo-Acilo-H_2O]$	$MS^3[(M-H)\rightarrow(M-H-Acilo)]^-$ $[M-acilo]^-$	$[M-Acilo-H_2O]^-$	$[Cin-H]^-$
*tri***S**inapoil**g**enc	959	735 (Sin)	529 (2 Sin)	511 (Sin)	529 (Sin)	223
*di***S**inapoil**F**eruloil**g**enc	929	705 (Sin)	499 (2 Sin)	481 (Sin)	499 (Sin)	
*di***S**inapoil**C**afeoil**g**enc	915	691 (Sin)	485 (2 Sin)	467 (Sin)	485 (Sin)	223
*di***S**inapoil**M**etoxi**C**afeoil**g**enc	945	721 (Sin)	515 (2 Sin)	497 (Sin) 529 (Metoxicaf)	515 (Sin)	223
(Outros)						
Sinapoil**C**afeoil**F**eruloilgenc	885	723 (Caf)	499 (Caf; Sin)	499 (Sin)		223
Sinapoil*di***F**eruloil**g**enc	899	705(Fer) 675 (Sin)			499 (Sin) 529 (Fer)	223

Legenda. $^{0,2}X$: Fragmento correspondente à perda parcial do açúcar, Cin: Ácido cinâmico, Glu: Glucose, Genc: Genciobiose, Sin: Sinápico, Fer: Ferúlico, Caf: Cafeico, Metoxicaf: Metoxicafeico.

2.8. Caracterização do potencial antioxidante dos compostos polifenólicos

As células com metabolismo aeróbio lidam continuamente com substâncias oxidantes de muitas origens e neutralizam os seus efeitos com um intricado conjunto de antioxidantes (148). Quando o equilíbrio redox é deslocado a favor dos oxidantes celulares gera-se stress oxidativo. Os factores indutores do stress oxidativo podem ser separados em endógenos (inflamação, reacções auto-imunes, desregulação do metabolismo, isquémia) e exógenos (microrganismos, radiação electromagnética e stress induzido por xenobióticos ou factores mecânicos). As espécies reactivas formadas incluem radicais livres como o óxido nítrico ($^{\bullet}NO$), o hidroxilo ($^{\bullet}OH$) e o superóxido ($O_2^{\bullet-}$) e espécies não radicalares como o peróxido de hidrogénio (H_2O_2), o ácido hipocloroso (HOCl) e o ácido peroxinitroso (HNOOH). As fontes de espécies reactivas mais importantes são a fosforilação oxidativa, o metabolismo pelo citocromo P450 e a activação de células inflamatórias.

Para se proteger contra o stress oxidativo os sistemas biológicos usam diversos mecanismos. Estes compreendem um grande número de moléculas antioxidantes de baixo peso molecular, que previnem a iniciação dos danos oxidativos ou limitam a sua propagação, enzimas que convertem e destoxificam as espécies reactivas ou que reparam o stress oxidativo quando ele ocorre e mecanismos que reencaminham as moléculas danificadas para destruição e substituição. As defesas antioxidantes endógenas são sobretudo enzimáticas e incluem a superóxido dismutase (SOD), a glutationa peroxidase (GPx) e a catalase (CAT). Um dos antioxidantes não enzimáticos mais importantes é a glutationa que pode captar directamente as espécies reactivas ou servir de cofactor a enzimas antioxidantes como a GPx.

Além das defesas antioxidantes endógenas, existem compostos exógenos como os compostos polifenólicos que podem exercer actividade antioxidante (149, 150).

2.8.1. Formação de espécies reactivas e interacção com as defesas antioxidantes endógenas

No metabolismo aeróbio formam-se diversas espécies reactivas de oxigénio (ROS), entre as quais o $O_2^{\bullet-}$ que está quase sempre na origem da formação das restantes. A SOD catalisa a reacção de dismutação do $O_2^{\bullet-}$ em H_2O_2, numa reacção dependente do pH, sendo muito importante no controlo dos níveis basais de $O_2^{\bullet-}$ (reacção 1, Figura 30). O H_2O_2 é também potencialmente oxidante, sendo eficientemente desactivado pela GPx e pela CAT. Contudo, na presença de vestígios de metais de

transição (principalmente ferro e cobre), pode produzir-se $^{\bullet}OH$, por reacções de Fenton (reacção 2). Nesta via a quantidade de metais redutores pode ser muito pequena, pois o próprio $O_2^{\bullet-}$ ou outros redutores "reciclam" o metal oxidado à forma reduzida activa (reacção 3). O $^{\bullet}OH$ é muito reactivo, o que implica uma baixa selectividade e distância de difusão e incapacidade de se tornar num mensageiro celular. Pelo contrário, a reacção do $^{\bullet}OH$ com os ácidos nucleicos, lípidos e proteínas pode causar danos celulares. O H_2O_2 é também o substrato da enzima mieloperoxidase que, na presença de iões cloreto, catalisa a conversão do $O_2^{\bullet-}$ numa espécie mais reactiva, o HOCl (ou o hipoclorito, OCl^-) que é simultaneamente oxidante e clorante (reacção 4) (151). O HOCl reage com o $O_2^{\bullet-}$ originando $^{\bullet}OH$ (reacção 5) (152). Dada a elevada reactividade do HOCl, em condições fisiológicas a sua concentração é muito baixa. Por essa razão, excepto quando há fagócitos activados, em que também se formam grandes quantidades de $O_2^{\bullet-}$, esta reacção não contribuirá para a produção de $^{\bullet}OH$.

Uma via metabólica independente, envolvendo as sintetases do óxido nítrico e a L-arginina conduz à formação do radical óxido nítrico ($^{\bullet}NO$). A convergência das vias metabólicas do $O_2^{\bullet-}$com o $^{\bullet}NO$ dá origem ao peroxinitrito ($HNOO^-$) (reacção 6) e espécies reactivas de azoto que dele derivam (RNS). A formação do $HNOO^-$ depende do equilíbrio entre a produção de $O_2^{\bullet-}$ e de SOD por um lado e da síntese e consumo de $^{\bullet}NO$ por outro (153). Em condições patofisiológicas em que a produção de $^{\bullet}NO$ é estimulada, a formação de $HNOO^-$ é significativa, sendo máxima quando os radicais $^{\bullet}NO$ e $O_2^{\bullet-}$ são produzidos em quantidades equivalentes (154).

Em pH fisiológico, o $HNOO^-$ (pKa = 6,8) existe em equilíbrio dinâmico com o seu ácido conjugado, o HNOOH (reacção 7). Nestas condições, a forma protonada decai rapidamente formando uma mistura de espécies de nitração e de oxidação muito tóxicas, algumas das quais envolvidas na reacção com o dióxido de carbono (CO_2) (155). A maior parte do HNOOH decompõe-se em nitrato (reacção 9) que é pouco reactivo. A reacção 8, em que se formam os radicais dióxido de azoto ($^{\bullet}NO_2$) e $^{\bullet}OH$ é minoritária.

A reacção do $HNOO^-$ com o CO_2 origina um intermediário instável, o carbonato nitrosoperóxido ($ONOOCO_2^-$, reacção 10), que é provavelmente a principal forma de eliminação de $HNOO^-$ *in vivo* (156). O conjugado formado na reacção 10 tem um tempo de vida muito curto e uma fracção substancial decompõe-se em $^{\bullet}NO_2$ e carbonato ($CO_3^{\bullet-}$) (reacção 11). Contudo, cerca de dois terços decompõe-se em nitrato (NO_3^-) e CO_2 (reacção 12). O $CO_3^{\bullet-}$ pode ser mais reactivo do que o $^{\bullet}NO_2$.

O $^{\bullet}NO_2$ pode também ser produzido pela reacção entre o $^{\bullet}NO$ e o oxigénio sem envolver o $O_2^{\bullet-}$ (reacção 13). Se a formação de $^{\bullet}NO_2$ for elevada, pode haver formação de trióxido de diazoto (N_2O_3) (reacção de equilíbrio 14). Este composto é um agente de

nitrosação muito potente, mas não é provável que se forme em grandes quantidades *in vivo*, com a possível excepção nas regiões hidrofóbicas de proteínas ou lípidos (como por exemplo nas membranas) (157).

O $^{\bullet}NO_2$ também pode ser formado por peroxidases, especialmente as mieloperoxidases, sem o envolvimento de $HNOO^-$ ou de oxigénio. As mieloperoxidases catalisam a oxidação do nitrito (NO_2^-) a $^{\bullet}NO_2$, usando o H_2O_2 como cofactor, sendo esta uma via importante para a nitração das proteínas (158).

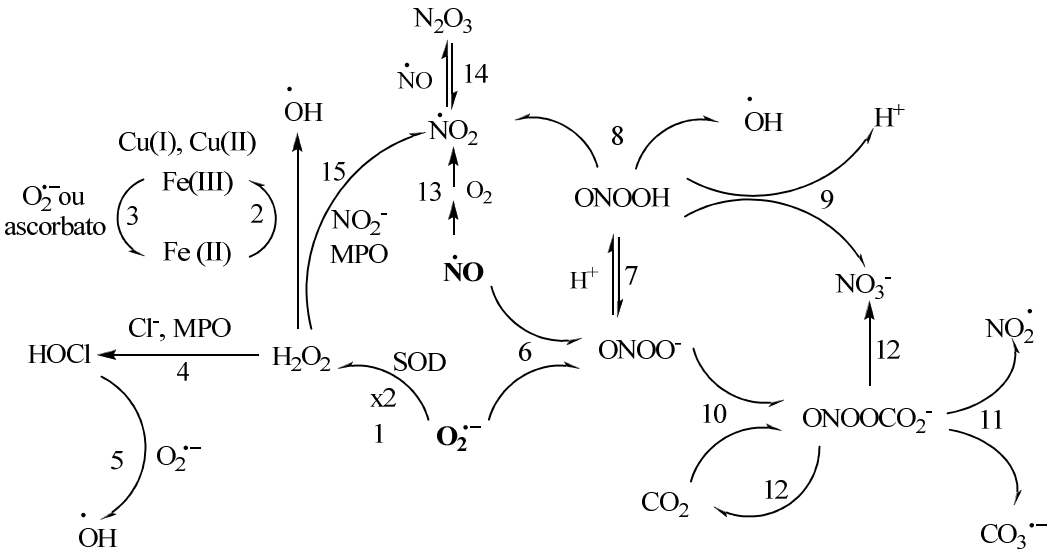


Figura 30. Principais espécies reactivas associadas com o stress oxidativo e nitrosativo, e vias de interacção entre as espécies [adaptada de (158)].

2.8.2. Actividade antioxidante dos compostos polifenólicos

O baixo potencial redox dos flavonóides permite-lhes reduzir radicais livres pela doação de um átomo de hidrogénio à espécie reactiva. Se o radical fenoxilo resultante for menos reactivo do que o radical inicial, as reacções de oxidação em cadeia são retardadas ou interrompidas. Alguns polifenóis, principalmente os que possuem um grupo catecol (3,4-di-hidroxilo), conseguem quelatar iões metálicos de transição, impedindo que estes exerçam acções pró-oxidantes (71).

A capacidade antioxidante dos compostos fenólicos é determinada pela sua estrutura, particularmente a facilidade com que um átomo de hidrogénio de um hidroxilo aromático pode ser doado a um radical livre e a capacidade do composto aromático suportar o electrão desemparelhado por deslocalização electrónica (159). Nos flavonóides, (Figura 31) as características estruturais mais importantes para o seu

potencial antioxidante são a presença de uma dupla ligação entre os carbonos 2 e 3 em conjugação com a função oxo no carbono 4 do anel C, a presença de um grupo catecol no anel B e a presença de um hidroxilo no carbono 5 em conjugação com a função oxo no carbono 4. Em particular o grupo catecol no anel B tem uma maior capacidade de doar electrões, e por essa razão, pode exercer uma actividade de captação de radicais livres muito forte (160).

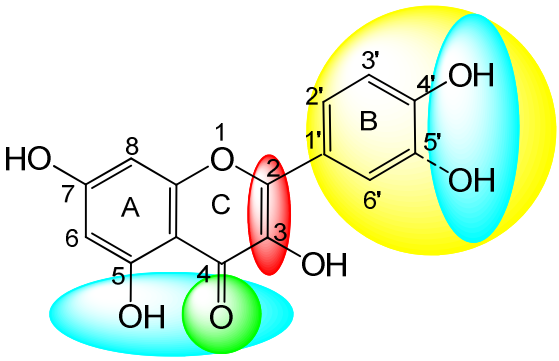


Figura 31. Estrutura química de flavonóides mostrando as características que definem o seu potencial antioxidante [adaptada de (161)].

Nota: Se o hidroxilo no carbono 5' (ligação a tracejado) existir trata-se da quercetina, senão trata-se do campferol.

O número total de grupos hidroxilo também pode ser determinante na actividade antioxidante.

Algumas das características essenciais para a eficácia dos flavonóides como antioxidantes (número e posição dos hidroxilos livres) podem ser responsáveis pelo seu potencial pró-oxidante (162).

Os ácidos hidroxicinâmicos têm algumas características estruturais semelhantes às descritas para os flavonóides que podem ser importantes para o seu potencial antioxidante. Por exemplo, o ácido cafeico tem um grupo catecol, uma ligação dupla na cadeia lateral conjugada com o anel fenólico e um grupo carbonilo que permitem a estabilização do radical fenoxilo (163).

Além da actividade antioxidante directa, os flavonóides podem contribuir para diminuir o stress oxidativo pela protecção ou optimização dos compostos antioxidantes endógenos ou pela indução de enzimas antioxidantes (159).

Actualmente pensa-se que os efeitos celulares dos flavonóides são mediados pelas suas interacções com proteínas específicas, fulcrais nas cascatas de sinalização intracelular (161). As acções de inibição ou de indução destas vias afectam profundamente as funções celulares alterando o estado de fosforilação de moléculas alvo e modulando a expressão dos genes.

Os flavonóides podem interagir com as mitocôndrias, interferir com as vias do metabolismo e/ou inibir a expressão de moléculas de adesão. Os efeitos celulares dos flavonóides dependem, em ultima análise, do grau em que se associam às células, pelas interacções na membrana ou pela absorção para o citosol (161).

2.9. ADME dos Flavonóides

O estudo da ADME (absorção, distribuição, metabolismo e excreção) dos flavonóides é crucial para poder avaliar a sua biodisponibilidade, as transformações que sofrem no organismo e que podem ser determinantes da mesma, o acesso que têm aos potenciais locais alvo, e as principais vias de excreção. O conhecimento destes fenómenos é fundamental para o entendimento da acção biológica destes compostos presentes na dieta, tanto localmente no tubo gastrintestinal, como após distribuição no organismo.

Devido ao grande número de compostos deste grupo e à variabilidade das suas estruturas nos diversos extractos vegetais, os estudos de ADME são ainda escassos e sobretudo incompletos. Nesta secção, a ADME dos flavonóides será abordada de forma genérica, numa perspectiva do entendimento das modificações que o organismo provoca nestes compostos e que condicionam a sua biodisponibilidade e acção biológica.

Estando os flavonóides presentes nas plantas essencialmente na forma de conjugados glicosídeos, a primeira e determinante alteração que genericamente sofrem no organismo verifica-se no tubo gastrintestinal, principalmente no intestino, onde as glucosidases operam a sua hidrólise, com libertação das geninas. A lactase florizina hidrolase (LPH), uma β-glicosidase membranar que se encontra ligada no lúmen intestinal dos mamíferos, além de hidrolisar a lactose, é capaz de hidrolisar eficazmente uma vasta gama de heterósidos de flavonóis e de isoflavonas. A LPH tem uma elevada afinidade para os heterósidos de glucose. Após a hidrólise do heterósido, a genina mais hidrofóbica pode difundir-se para as células epiteliais, enquanto que a glucose é transportada pelo transportador de glucose dependente do sódio (SGLT1) que nos enterócitos se encontra na proximidade da enzima LPH (164, 165).

As geninas livres, sendo mais lipofílicas que os precursores conjugados, são mais eficazmente absorvidas nos enterócitos por difusão passiva. Estas reacções de Fase I, no intestino, condicionam assim fortemente a absorção dos flavonóides veiculados na dieta. Várias revisões de estudos publicados sobre o assunto evidenciam a importância deste fenómeno (166, 167).

Para além da actividade das glicosidases presentes no intestino delgado, responsáveis pela metabolização por reacções de fase I (desglicosilação), nos enterócitos ocorrem reacções metabólicas de fase II nas geninas resultantes, incluindo a glucuronidação, sulfatação e *O*-metilação de grupos catecólicos, por enzimas como a UDP-glucuroniltransferase, fenol sulfotransferase e catecol-*O*-metiltransferase. A corroborar a importância destas reacções de metabolização ocorridas no intestino estão os estudos que demonstram que, após administração oral dos derivados glicosídeos, na corrente sanguínea circulam preponderantemente os conjugados glucuronídeos, sulfatos, ou metilados, estando os conjugados glicosídeos e as respectivas geninas ausentes ou em muito baixos níveis (166). O intestino delgado é assim um órgão muito activo na glucuronidação dos flavonóides, sendo também importante na metilação, o que faz com que seja considerado o órgão que realiza metabolismo de primeira passagem destes compostos. O fígado é muito importante na sulfação, mas também na glucuronidação e metilação adicionais (Figura 32) (168).

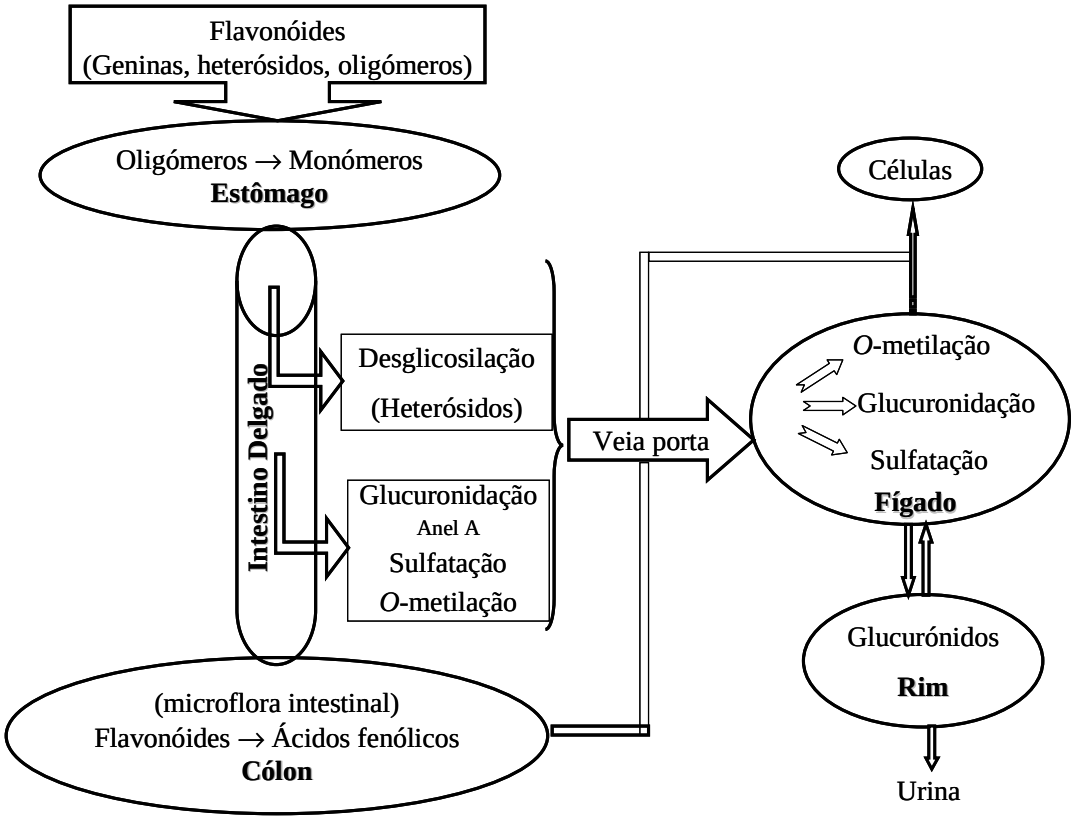


Figura 32. Resumo da formação dos metabolitos e conjugados no tracto gastrointestinal e hepático [adaptada de (169)]

Os flavonóides que não são metabolizados e/ou absorvidos no intestino delgado, bem como os metabolitos conjugados excretados na bile podem, no cólon, ser degradados pelas enzimas da microflora aí presentes a ácidos fenólicos simples. A degradação completa ocorre pela quebra do anel da pirona produzindo-se ácidos fenilacéticos ou fenilpropanóicos e outros sub-produtos inertes. Estes compostos podem ser absorvidos e posteriormente metabolizados no fígado (169).

De referir que, ainda no ambiente de baixo pH do estômago, pode ocorrer a quebra dos flavonóides oligoméricos como as procianidinas.

A correcta avaliação da biodisponibilidade dos flavonóides é uma tarefa muito árdua, pois varia de composto para composto e vários factores interferem no resultado final. Para além das reacções hidrolíticas de fase I, da absorção das geninas nos enterócitos e subsequentes reacções de fase II (glucuronidações e metilações essencialmente), há que entrar em linha de conta com os efeitos das bombas de efluxo presentes nos enterócitos e que desempenham um importante papel na excreção dos compostos, livres ou conjugados, no lúmen intestinal (170). Os heterósidos podem entrar nas células intestinais mas são facilmente exportados por transportadores de membrana como o MRP-2 (Multidrug resistance-associated protein-2) (165).

Na Figura 33 estão esquematizadas as vias de absorção de flavonóides no intestino delgado.

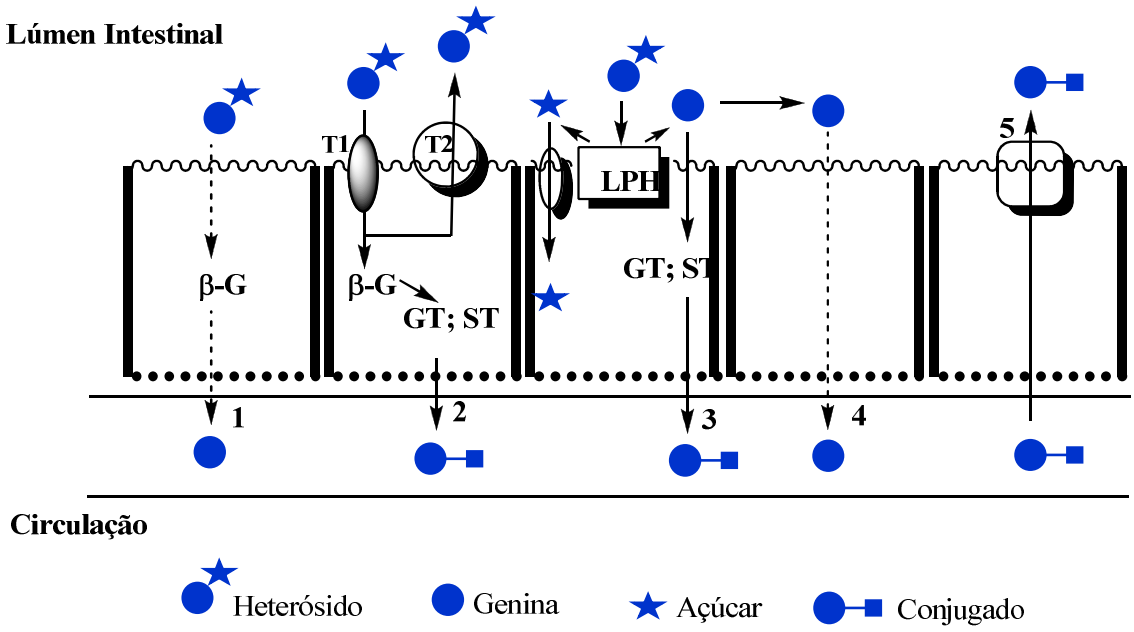


Figura 33. Vias de absorção de metabolitos de flavonóides no intestino delgado [adaptada de (165)].

Acresce ainda o aspecto relevante das interacções que podem ocorrer entre os flavonóides ingeridos na dieta e os múltiplos componentes da mesma, sendo impossível prever a interferência que possam ter na absorção daqueles e respectiva biodisponibilidade. Esta é uma área onde se desenvolve muita investigação.

Dois dos principais flavonóides obtidos pela dieta são a quercetina e o campferol. O campferol, o principal flavonol encontrado em espécies de *B. oleracea* contribui significativamente para a quantidade de flavonóides absorvidos. Nos seres humanos, o campferol representa entre 25-33% do valor médio de flavonóides absorvidos, com valores estimados de 6 a 10 mg por dia nos Estados Unidos da América (44).

Num estudo realizado com ratos verificou-se que após administração oral de quercetina, 20% foi directamente absorvida, 30% foi excretada nas fezes sem sofrer qualquer modificação e a restante foi metabolizada a vários compostos incluindo CO_2 e ácidos fenólicos (171).

Sistematizando, e de uma forma genérica, o metabolismo dos flavonóides verifica-se essencialmente por reacções de Fase I e Fase II:

1. Reacções de Fase I

As reacções de Fase I, conforme explanado acima, podem ser reacções de hidrólise, mediadas no tubo gastrintestinal pelas glucosidases. Especialmente após absorção e passagem ao fígado, os flavonóides podem ser metabolizados por enzimas do citocromo P450, sendo as reacções de oxidação as mais prováveis, consistindo em hidroxilações e desmetilações. Pensa-se que as isoenzimas CYP1A têm um papel importante na biotransformação de flavonóides (172).

Como a expressão de isoformas das enzimas CYP varia muito entre indivíduos e algumas delas são polimórficas, pode especular-se que diferenças individuais na capacidade de transformação de flavonóides pode tornar certas pessoas mais ou menos refractárias às suas acções (173).

Os flavonóides também podem sofrer metabolismo oxidativo por peroxidases e polifenol oxidases (174, 175).

Apesar do metabolismo oxidativo dos flavonóides poder facilitar a sua eliminação, pode também ser considerada uma via de activação. Por exemplo, a oxidação da galangina a campferol que pode prosseguir para quercetina, resulta na formação de moléculas progressivamente mais activas no que diz respeito às propriedades antioxidantes (176).

A oxidação dos flavonóides pode originar produtos pró-oxidantes ou alquilantes do tipo quinonas ou quinóides (Figura 34) (174, 175). Esta conversão pode ser acompanhada pela formação de níveis citotóxicos de ROS, sobretudo para concentrações elevadas de flavonóides (162).

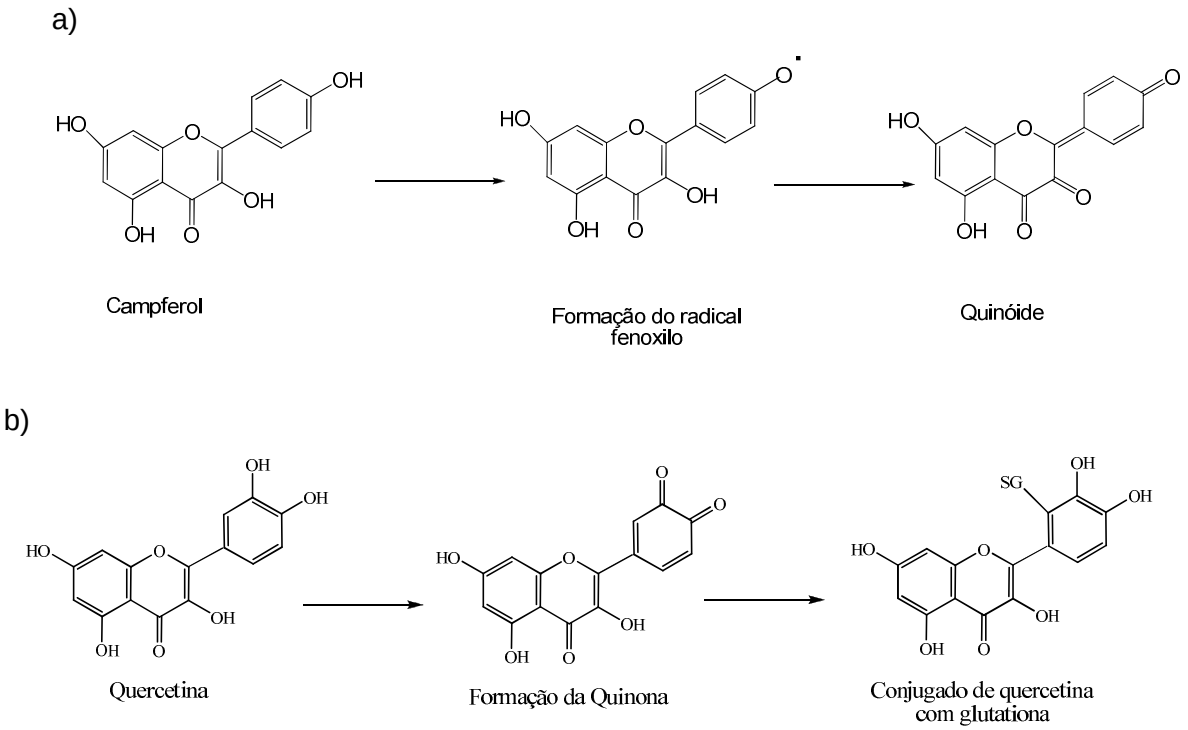


Figura 34. Oxidação de flavonóides na presença de peroxidases.

a) Oxidação do campferol a um composto quinóide (177); b) Oxidação da quercetina a uma quinona e posterior conjugação com a GSH (161).

A formação de quinonas e produtos quinóides pode dever-se à auto-oxidação dos flavonóides na presença de oxigénio em sistemas que contenham metais de transição como o cobre e o ferro. Embora *in vivo* a maior parte dos metais de transição se encontre sequestrada, em situações patológicas como por exemplo em tecidos lesionados (aterosclerose) e de sobrecarga hepática como na doença de Wilson, é possível que a libertação de iões metálicos seja suficiente para catalisar reacções de oxidação de flavonóides (178).

2. Reacções de Fase II

Conforme também já mencionado atrás, os flavonóides sofrem reacções de conjugação de uma forma extensa. Este facto não é surpreendente, pois sendo

polifenois, os hidroxilos livres podem ser prontamente conjugados, sendo a glucuronidação e metilação reacções abundantemente operadas no enterócito e subsequentemente no fígado, após absorção, bem como as reacções de sulfatação que são essencialmente abundantes neste órgão.

Conjugados tióis têm já sido também caracterizados. Assim, as quinonas e quinóides com origem na oxidação dos flavonóides podem formar conjugados com tióis intracelulares como a cisteína, a GSH e a N-acetilcisteína (179). Destes tióis, a cisteína apresenta maior afinidade, mas, as concentrações fisiológicas de GSH muito superiores fazem com que os conjugados glutationilo sejam os predominantes. Também é possível que exista a adição covalente destas quinonas a grupos sulfidrilo proteicos, o que pode ser particularmente importante, uma vez que muitas enzimas contêm resíduos de cisteína nos locais catalíticos ou de regulação (180).

A conjugação das quinonas com a GSH nem sempre resulta na sua destoxificação. Por um lado, a diminuição dos níveis de GSH celular resultante é prejudicial. Por outro lado, só quando a conjugação com a GSH se encontra associada à subsequente exportação dos conjugados das células, a destoxificação é a consequência predominante (181). Uma vez no meio extracelular e/ou na circulação, os conjugados podem interagir com outras células capazes de acumular estes metabolitos, como por exemplo as células do rim. A acumulação de conjugados nas células do rim pode ser citotóxica, da mesma forma que para os conjugados glutationilo da 3,4-metilenodioximetanfetamina (MDMA), os quais expressam um forte efeito nefrotóxico por reacções do ciclo redox na membrana apical das células renais proximais tubulares (182). As consequências para as células, resultantes da formação de derivados da glutationa com os compostos quinónicos dos flavonóides, constitui um desafio adicional na avaliação da influência do metabolismo destes compostos na sua actividade biológica.

Também é possível que, dado o seu peso molecular relativamente elevado (acima de 500), os conjugados formados sejam excretados na bílis. Assim, os conjugados flavonóide – glutationa podem ser excretados no duodeno, onde o ácido glutâmico e a glicina são sequencialmente removidos por enzimas da bílis, ou por microrganismos no tracto gastrintestinal, levando à formação de conjugados cisteinil. Estes conjugados podem ser reabsorvidos do tracto gastrintestinal para a circulação, e depois ser absorvidos por células ou ser excretados pela urina após a adição de um grupo acetilo no rim (169).

Conforme já dito, o grande número de compostos flavonóides presentes nos extractos vegetais com interesse na alimentação humana faz com que os estudos de ADME estejam ainda longe de estar completos, contribuindo para a dificuldade da sua

realização vários factores interferentes. Mesmo assim, a absorção e a biotransformação destas moléculas são os fenómenos mais bem estudados até ao momento. Sobre a distribuição nos tecidos, existem alguns estudos realizados em animais de laboratório, para algumas moléculas administradas quase sempre de forma individual, mas cujos resultados não nos permitem fazer uma generalização para os flavonóides como um grupo.

Para avaliar a distribuição da quercetina nos tecidos, de Boer e os seus colaboradores, 2005, fizeram vários ensaios com ratos e porcos alimentados com diferentes quantidades de quercetina e com diferentes períodos de exposição (183). Neste estudo verificou-se que a quercetina e os seus metabolitos têm uma distribuição generalizada por todo o organismo, sendo a sua acumulação em cada tecido dependente da quantidade e do período de administração. Por exemplo, ratos alimentados durante 11 semanas com uma dieta contendo 1% de quercetina acumularam 15,3 nmol/g no tecido pulmonar, mas noutros órgãos como o cérebro, a gordura amarela e o baço, a quantidade acumulada foi muito menor. Os porcos alimentados durante 3 dias com uma dieta contendo 500 mg de quercetina / kg de peso acumularam 5,87 nmol de quercetina /g no tecido hepático e 2,51 nmol de quercetina /g no tecido renal, enquanto que o cérebro, o coração e o baço acumularam quantidades inferiores (183).

O destino da maior parte dos metabolitos conjugados é a excreção renal e/ou biliar, apesar de alguns entrarem nas células e tecidos (169). A análise por LC dos flavonóides e dos seus produtos de degradação na urina e nas fezes evidenciou a existência de compostos metilados, hidroxilados, *O*-metilados, sulfatados e glucurónidos como produtos da transformação no fígado e na flora intestinal (184). Os estudos de excreção dos flavonóides revestem-se também da maior importância, são passíveis de ser feitos em voluntários humanos, e técnicas modernas como LC-MS/MS constituem uma excelente forma de elucidação estrutural dos metabolitos eliminados. No entanto, as dificuldades são também grandes, pois são múltiplos os possíveis metabolitos de eliminação dos flavonóides, conforme demonstra o estudo de eliminação da quercetina na urina, tendo sido identificados 23 metabolitos (185)

É ainda importante ter em mente que estes compostos, dado que são conjugados nos enterócitos e podem ser depois excretados para o lúmen intestinal ou ser absorvidos, passar ao fígado e excretados na bile, podem ser em alguma extensão eliminados nas fezes. Além disso, os metabolitos formados no cólon podem ser absorvidos e excretados na urina ou serem em parte eliminados nas fezes.

Não sendo os estudos de ADME dos flavonóides encarados no âmbito desta dissertação, a abordagem simplista destes fenómenos pretende dar uma ideia da importância determinante que resulta na expressão da sua acção biológica.

2.10. Avaliação do potencial antioxidante da couve tronchuda em culturas de hepatócitos primários de rato

Os ensaios de avaliação de actividade antioxidante realizados em sistemas químicos não reflectem as condições fisiológicas celulares e não têm em linha de conta a bio-disponibilidade e metabolismo dos extractos estudados.

Além disso, os mecanismos de acção dos antioxidantes nas células não se limitam à captação de espécies reactivas, ao contrário dos ensaios químicos (186, 187). Algumas das consequências do stress oxidativo nas células encontram-se esquematizadas na Figura 35 (188).

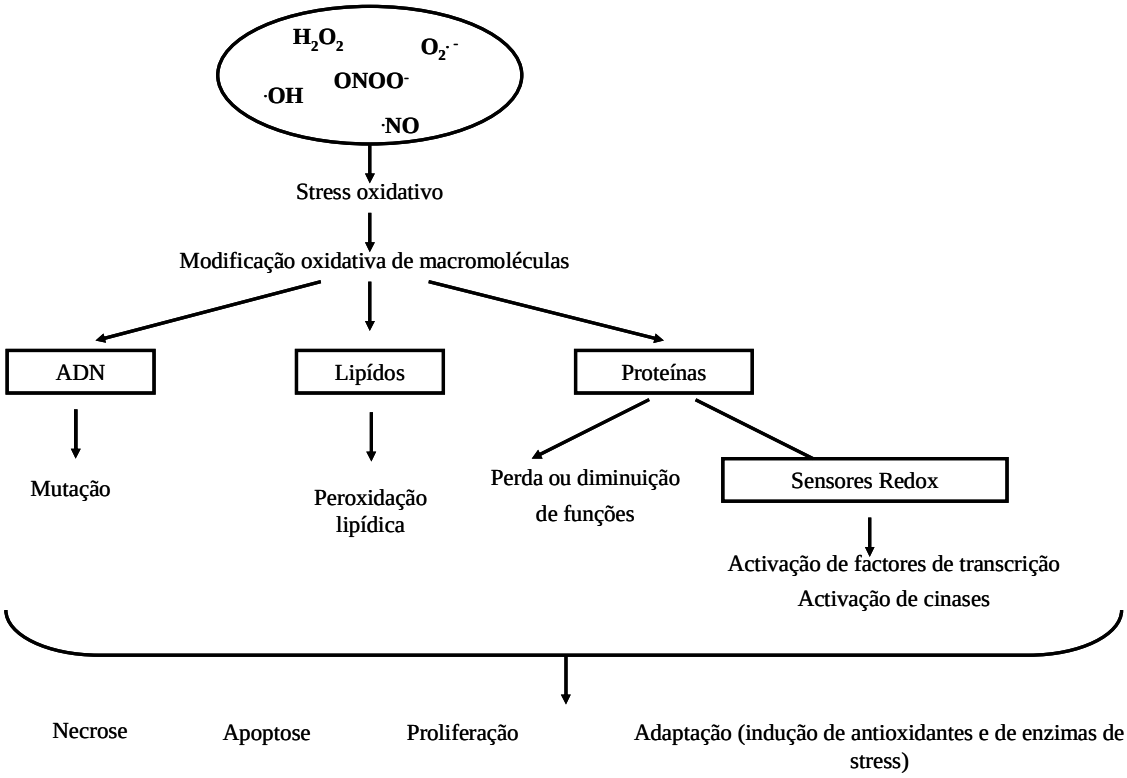


Figura 35. Consequências do stress oxidativo numa célula [adaptada de (188)].

Dada a importância do fígado nos processos de biotransformação de xenobióticos, e os elevados níveis de stress oxidativo a que as suas células podem estar sujeitas, os hepatócitos, em suspensão ou em cultura, são um modelo relevante para a avaliação do potencial antioxidante de extractos em sistemas celulares. A cultura celular tem algumas vantagens relativamente às suspensões, uma vez que os hepatócitos têm tempo de recuperar do stress induzido durante o isolamento, podem ser mantidos por períodos mais longos e podem estudar-se efeitos de compostos que requerem a

organização das células (187, 188). Tipicamente, os ensaios *in vitro* permitem responder a questões ao nível celular e molecular (toxicologia mecanística) (189).

O modelo de cultura de hepatócitos primários de rato tem sido usado por muitos autores para avaliar a hepatoprotecção de extractos de plantas, usando substâncias modelo para induzir stress oxidativo entre as quais o *t*-butilidroperóxido (t-BOOH), etanol, tetracloreto de carbono (CCl_4) e o H_2O_2 (190-194). O paraquato (PQ) também é um modelo comum usado em culturas celulares (195). Estes compostos têm diferentes mecanismos de toxicidade, mas pensa-se que todos exercem os seus efeitos induzindo stress oxidativo.

Tem-se verificado que os extractos vegetais ou alguns dos seus componentes podem ser protectores ou tóxicos, dependendo das concentrações de extracto utilizadas, das células estarem ou não sujeitas a stress e do tipo de stress induzido (196). Os extractos de plantas ricos em compostos polifenólicos demonstram quase sempre ser hepatoprotectores. Por exemplo, o extracto de alcachofra (*Cynara scolymus*), rico em ácido cafeico, ácido clorogénico, cinarina e cinarósido reduz a peroxidação lipídica, a perda de GSH e morte celular em hepatócitos expostos ao t-BOOH e ao H_2O_2, o mesmo se verificando para cada um dos constituintes atrás mencionados (190, 197). Os extractos aquosos de *Euonymus alatus* protegem os hepatócitos contra os danos induzidos pelo H_2O_2 (192), os extractos da pele de ameixa preta indiana (*Syzygium cumini*), ricos em antocianinas, protegem os hepatócitos contra o CCl_4 e aumentam a actividade da GPx (194) e os extractos de avelãs e nozes (aquosos, metanólicos e lipofílicos, usando o etilacetato como solvente) protegem os hepatócitos contra a toxicidade do H_2O_2, (com excepção do extracto lipofílico de avelã) (191).

Contudo, nem sempre os extractos têm o efeito protector esperado: um extracto de *D. pinnatifida* não demonstrou ter efeito protector em hepatócitos tratados com t-BOOH, etanol, CCl_4 e lipopolissacarídeo (LPS) (193). No mesmo trabalho Rodeiro e os seus colaboradores testaram outros extractos (*Mangifera indica*, *Erythroxylum minutifolium*, *Erythroxylum confusum*, *Thalassia testudinum* e *Dictyota pinnatifida*) e também mangiferina, o fenol maioritário do extracto de *M. indica*, tendo verificado um efeito protector variável, dependendo do agente agressor e do extracto testado.

Nos ensaios realizados com compostos fenólicos, também se observa que estes, por si só, podem ser citotóxicos para as células, quando se utilizam concentrações elevadas. As catequinas presentes no chá preto podem ser citotóxicas, provavelmente por alterarem o potencial da membrana mitocondrial e por levarem à formação de ROS (198), a quercetina pode ligar-se ao ADN, (199) e o próprio campferol, em concentrações na ordem de 1 mM causa citotoxicidade aos hepatócitos ao fim de 2 horas de exposição (177).

Para avaliar os efeitos protectores dos extractos de couve tronchuda nos hepatócitos, escolheu-se como agente indutor de stress oxidativo o PQ. Os ciclos de oxidação-redução do paraquato levam à produção de $O_2^{\cdot -}$ (Figura 36) que, se não for captado, pode originar danos celulares. Além disso, o consumo de NADPH pelas diaforases celulares na redução do catião PQ^{2+} ao catião radicalar $PQ^{\cdot +}$ pode impedir processos bioquímicos importantes dependentes deste redutor (200).

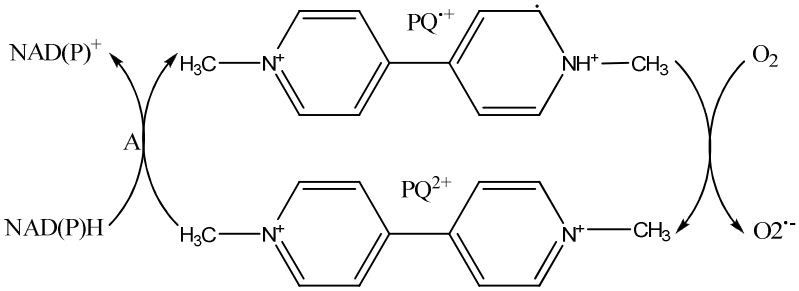


Figura 36. Ciclo Redox do Paraquato.

A. Diaforases celulares

Os extractos com potencial antioxidante podem impedir os danos causados pelo PQ nas células. A avaliação de parâmetros de viabilidade celular, como a LDH (lactato desidrogenase), parâmetros dependentes do estado redox da célula como o ATP e a glutationa e a peroxidação lipídica e também a acção do extracto na via de sinalização do NFkB, permitirá avaliar a capacidade de protecção dos extractos de couve tronchuda.

3. OBJECTIVOS DA DISSERTAÇÃO

1. Caracterização do perfil metabólico de *B. oleracea* var. *costata* (couve tronchuda) usando extractos aquosos obtidos por decocção. As partes da planta a caracterizar constituem as sementes, as plântulas (sementes germinadas), as folhas internas, as folhas externas e as inflorescências. As classes de compostos a caracterizar são:

   1.1. Compostos polifenólicos
   1.2. Ácidos orgânicos

   Para além destas classes, nos extractos de folhas internas e de folhas externas, que são as partes da planta habitualmente consumidas, pretende-se ainda caracterizar:

   1.3. Aminoácidos livres
   1.4. Compostos voláteis (isotiocianatos, terpenóides, norisoprenóides e compostos derivados dos ácidos gordos)

2. Avaliação de variações no perfil de compostos atrás referidos dos extractos de folhas internas e de folhas externas de couve tronchuda com:
   2.1. Regime de fertilização (sem fertilizantes, fertilização biológica, fertilização convencional)
   2.2. Época de colheita

3. Caracterização do perfil polifenólico de rebentos caulinares de couve tronchuda obtidos por micropropagação, usando extractos aquosos. Avaliação do efeito da utilização de diferentes meios de cultura e fitoreguladores neste perfil.

4. Avaliação da capacidade de sequestração e de biotransformação de compostos polifenólicos pela larva de *P. brassicae*, alimentada com couve tronchuda, através da caracterização de extractos aquosos de larva, obtidos por decocção.

5. Avaliação do potencial antioxidante das matrizes atrás referidas, usando as espécies DPPH, superóxido, hidroxilo, ácido hipocloroso, óxido nítrico e peroxinitrito.

6. Avaliação da actividade antimicrobiana de um extracto aquoso de inflorescências de couve tronchuda.

7. Avaliação da hepatoprotecção de um extracto aquoso de folhas externas de couve tronchuda em hepatócitos primários de rato expostos ao paraquato.

PARTE II

SECÇÃO EXPERIMENTAL

4. SECÇÃO EXPERIMENTAL

4.1. Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) seeds: Phytochemical characterization and antioxidant potential.

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Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) seeds: Phytochemical characterization and antioxidant potential

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Abstract

Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) seeds were studied for their chemical composition and antioxidant capacity. Thirteen phenolic compounds were characterized and quantified by reversed-phase HPLC-DAD-MS/MS-ESI and HPLC-DAD, respectively: two sinapoylgentiobiose isomers, three sinapoylglucose isomers, kaempferol-3-(sinapoyl)sophorotrioside-7-glucoside, sinapoylcholine, kaempferol-3,7-diglucoside-4′-(sinapoyl)glucoside, three disinapoylgentiobiose isomers, 1,2,2′-trisinapoylgentiobiose and 1,2-disinapoylglucose. Seven organic acids (aconitic, citric, ascorbic, malic, quinic, shikimic and fumaric acids) were also identified and quantified by HPLC-UV. The aqueous extract of tronchuda cabbage seeds was investigated for its capacity to act as a scavenger of DPPH· radical and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid), exhibiting antioxidant capacity in a concentration-dependent manner against all radicals. These results may be attributed to the high content of hydroxycinnamic derivatives and ascorbic acid.
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Keywords: Tronchuda cabbage seeds; *Brassica oleracea* L. var. *costata* DC; Phenolic compounds; Organic acids; Antioxidant capacity

1. Introduction

Brassicaceous plants represent one of the major vegetable crops grown, worldwide, constituting an important part of a well balanced diet. *Brassica oleracea* is a native of the Mediterranean region and southwestern Europe, extending northward to southern England (Vaughan and Geissler, 1997a). It is easy to grow in cold weather, requires moist soil and can tolerate maritime exposure. Horticultural selection within the species has led to the development of a number of cultivars and, although essentially temperate, *B. oleracea* forms are today grown for food everywhere that plants can grow (Vaughan and Geissler, 1997a). Tronchuda cabbage (*B. oleracea* L. var. *costata* DC) is especially popular in Portugal, having a determinant role in the Portuguese diet and agricultural systems.

An increasing amount of evidence shows the need for a constant supply of phytochemical-containing plants to achieve optimal health benefits. This is ascribed to the fact that different plants have distinct compound contents, with several structures, thus offering different protective mechanisms at different levels (Chu, Sun, Wu, and Liu, 2002; Ninfali and Bacchiocca, 2003). Among phtyochemicals, phenolic compounds and organic acids may exert a protective role against various diseases, due to their antioxidant potential (Silva et al., 2004). In addition, these compounds are known to play an important role in maintaining fruit and vegetable quality, contributing to their organoleptic characteristics (Vaughan and Geissler, 1997 b), and have also been used for the quality control of several matrices

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(Valentão et al., 2005; Valentão, Andrade, Areias, Ferreres, and Seabra, 1999).

The phenolic composition of tronchuda cabbage leaves has already been reported: the external leaves were characterized by the presence of complex flavonol glycosides (Ferreres et al., 2005), while the internal ones exhibited both flavonol glycosides and hydroxycinnamic acid derivatives (Ferreres et al., 2006). The organic acids profile and the antioxidant capacity of external and internal leaves were also previously described (Ferreres et al., 2006; Vrchovska et al., 2006), with the external ones exhibiting higher antioxidant potential. However, nothing has been reported about tronchuda cabbage seeds. In fact, several studies with other *Brassica* species have reported the existence of phenolics in the seeds, namely phenolic acids and their derivatives (Baumert et al., 2005; Bouchereau, Hamelin, Lamour, Renard, and Larher, 1991; Li and El Rassi, 2002; Naczk, Amarowicz, Sullivan, and Shahidi, 1998), flavonoid glycosides (Baumert et al., 2005) and tannins (Naczk et al., 1998). These compounds have been considered as UV screens in young seedlings (Gitz, Liu, and McClure, 1998) and have been associated with seedling vigour, height and weight (Randhir and Shetty, 2003; Randhir and Shetty, 2005). Regarding *B. oleracea* seeds, previous studies with different varieties, other than *costata*, concerned its germination sensitivity to hypoxia (Finch-Savage, Côme, Lynn, and Corbineau, 2005), the effect of its film-coating to control insect pests (Ester, Putter, and Bilsen, 2003), the determination of glucosinolates (Kaoulla, MacLeod, and Gil, 1980; MacLeod, MacLeod, and Reader, 1989; Rangkadilok et al., 2002a, 2002 b), fatty acids (Ayaz et al., 2006) and sterols (Matsumoto, Shimizu, Asano, and Itoh, 1983).

Thus, the objectives of this study were to define the phenolic and organic acid compositions of tronchuda cabbage seeds and to evaluate their antioxidant potential. Phenolic profile was established by reversed-phase HPLC-DAD-MS/MS-ESI and HPLC-DAD analysis, while organic acids were determined by HPLC/UV. The antioxidant capacity was assessed by DPPH$^{\cdot}$ radical and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid)-scavenging assays.

2. Materials and methods

2.1. Standards and reagents

Malic, quinic, shikimic and fumaric acids were purchased from Sigma (St. Louis, MO, USA). Aconitic, citric, ascorbic and sinapic acids and kaempferol-3-*O*-rutinoside were from Extrasynthése (Genay, France). Methanol, formic and acetic acids were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA). DPPH, xanthine, xanthine oxidase (XO) grade I from buttermilk (EC 1.1.3.22), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), anhydrous ferric chloride ($FeCl_3$), ethylenediaminetetraacetic acid disodium salt (EDTA), ascorbic acid, trichloroacetic acid, thiobarbituric acid, deoxyribose, sodium hypochlorite solution with 4% available chlorine (NaOCl), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. (St. Louis, USA).

2.2. Samples

Tronchuda cabbages seeds were obtained from local farmers in Bragança, Northeast Portugal, in July 2005.

2.3. Sample preparation

An aqueous extract was used for the phytochemical characterization and in the antioxidant activity assays: ≈6.0 g of powdered tronchuda cabbage seeds were boiled for one hour in 600 ml of water and then filtered over a Büchner funnel. The resulting extract was lyophilized in a Labconco 4.5 Freezone apparatus (Kansas City, MO, USA) and a yield of ≈0.9 g was obtained. The lyophilized extract was kept in an desiccator, in the dark.

For the characterization and quantification of the phenolic compounds by HPLC-DAD-MS/MS-ESI and HPLC-DAD, respectively, the lyophilized extract was redissolved in water (100 mg/ml), ultra-sonicated, centrifuged and filtered (0.45 μm). For organic acids determination it was redissolved in 0.01 N sulphuric acid (100 mg/ml) prior to analysis by HPLC-UV.

2.4. HPLC-UV analysis of organic acids

The separation of the organic acids present in the seed lyophilized extract was carried out as previously reported, in a system consisting of an analytical HPLC unit (Gilson) with an ion exclusion column, Nucleogel® Ion 300 OA (300 × 7.7 mm) in conjunction with a column heating device set at 30 °C. Briefly, elution was carried out isocratically, at a solvent flow rate of 0.2 ml min^{-1}, with 0.01 N sulphuric acid (Ferreres et al., 2006). The detection was performed with a UV detector set at 214 nm.

Organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Malic and quinic acid were quantified together as malic acid. The average regression equations for aconitic, citric, ascorbic, malic, shikimic and fumaric acids were $y = 3.29 \times 10^7 x$, $y = 7.90 \times 10^7 x$, $y = 1.33 \times 10^7 x$, $y = 5.95 \times 10^7 x$, $y = 4.84 \times 10^9 x$ and $y = 1.05 \times 10^{10} x$, respectively. The detection limit values ranged from 0.01 to 1.67 μg/ml.

2.5. HPLC-DAD-MS/MS-ESI qualitative analysis of phenolic compounds

Chromatographic separations were carried out on a 250 × 4 mm, 5 μm particle size, RP-18 LiChroCART (Merck, Darmstadt, Germany) column protected with a

4 × 4 mm LiChroCART guard column. Elution was performed using acetic acid 1% (A) and methanol (B) as solvents, starting with 20% B and using a gradient to obtain 50% B at 30 min and 80% B at 37 min. In the MS/MS positive ionisation mode study, 1% formic acid was used instead of acetic acid, in order to enhance ionisation. The flow rate was 1 ml min^{-1} and the injection volumes varied between 10 and 50 μl.

The HPLC system was equipped with an Agilent 1100 Series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser and a G1315B photo-diode array detector controlled by ChemStation software (Agilent, v. 08.03). Spectroscopic data from all peaks were accumulated in the range 240–400 nm, and chromatograms were recorded at 330 nm. The mass detector was a G2445A Ion-Trap Mass Spectrometer equipped with an electrospray ionisation (ESI) system and controlled by LCMSD software (Agilent, v. 4.1.). Nitrogen was used as nebulizing gas at a pressure of 65 psi and the flow was adjusted to 11 l min^{-1}. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full scan mass covered the range from m/z 90 up to m/z 2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS data were acquired in the negative ionisation mode and in the positive ionisation mode for the study of compound **7**. MS^n data were achieved in the automatic mode on the more abundant fragment ion in MS^{n-1}. Table 1 shows the most frequent ions which characterize the fragmentation of the compounds. Other ions were found but they have not been included due to their low significance. The classical nomenclature for glycoconjugates was adopted to designate the fragment ions. The ions $^{k,l}X_j$ represent fragments still containing the aglycone, where j is the number of the interglycosidic bond broken, counted from the aglycone, and the k and l denote the cleavage within carbohydrate rings (Domon and Costello, 1988; Ferreres, Llorach, and Gil-Izquierdo, 2004; Hvattum and Ekeberg, 2003). For a better comprehension, data in Table 1 were grouped according to sinapoyl derivatives, relative to the number of acid molecules and, among these, by isomers. Data for sinapoyl derivatives from flavonoid glycosides were also included in this table.

2.6. HPLC-DAD quantitative analysis of phenolic compounds

Twenty microlitres of the seeds' lyophilized extract were analysed using an HPLC unit (Gilson) and a RP-18 LiChroCART (Merck, Darmstadt, Germany) column (250 × 4 mm, 5 μm particle size), under the conditions described for the qualitative analysis. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200–400 nm, and chromatograms were recorded at 330 nm. The data were processed on a Unipoint Software system (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Since standards of the compounds identified in the seeds' lyophilized extract were not commercially available, sinapic acid derivatives were quantified as sinapic acid and the kaempferol derivatives as kaempferol-3-*O*-rutinoside. The average regression equations for sinapic acid and kaempferol 3-*O*-rutinoside were $y = 1.71 \times 10^9 x$ and $y = 7.42 \times 10^8 x$, respectively. The detection limit was 5.9 μg/ml for sinapic acid and 4.4 μg/ml for kaempferol 3-*O*-rutinoside.

2.7. DPPH$^{\bullet}$ scavenging activity

The antiradical activity of the extracts was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc.), by monitoring the disappearance of DPPH$^{\bullet}$ at 515 nm, according to a described procedure (Ferreres et al., 2006; Vrchovska et al., 2006). For each extract, a dilution series composed of five different concentrations was prepared in a 96 well plate. The reaction mixtures in the sample wells consisted of 25 μl of aqueous extract and 200 μl of DPPH$^{\bullet}$ dissolved in methanol. The plate was incubated for 30 min at room temperature. Three experiments were performed in triplicate.

2.8. Evaluation of superoxide radical-scavenging activity

2.8.1. General

Antiradical activity was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc.), by monitoring the effect of the lyophilized extracts on the $O_2^{\bullet-}$-induced reduction of NBT at 562 nm.

2.8.2. Non-enzymatic assay

Superoxide radicals were generated by the NADH/PMS system according to a described procedure (Valentão et al., 2001a, 2001 b). All components were dissolved in phosphate buffer (19 mM, pH 7.4). Three experiments were performed in triplicate.

2.8.3. Enzymatic assay

Superoxide radicals were generated by the xanthine/xanthine oxidase (X/XO) system, following a described procedure (Valentão et al., 2001a, 2001 b). Briefly, xanthine was dissolved in NaOH (1 μM) and subsequently in phosphate buffer (50 mM) with EDTA (0.1 mM, pH 7.8), xanthine oxidase in EDTA (0.1 mM) and the remaining components in phosphate buffer (50 mM) with EDTA (0.1 mM, pH 7.8). Three experiments were performed in triplicate.

Table 1
R_t and most frequent ions which characterize the MS fragmentation of tronchuda cabbage seeds phenolic compounds

Compounds[a]		R_t (min)	MS^n, m/z(%)							
		Monosinapoyl derivatives								
			$[M-H]^-$				$MS^2[M-H]^-$			
							$[M-H-18]^-$	$[^{0,2}X_0-18]^-$	$[Sinp-H]^-$	$[Sinp-H-18]^-$
1	**SinpGentb**	9.0	547				529(100)	247(50)	223(4)	
3	**SinpGentb**	10.7	547				529(100)	247(60)	223(12)	
2	**SinpGlc**	9.7	385				367(17)	247(100)	223(2)	
4	**SinpGlc**	11.7	385				367(14)	247(100)	223(3)	
5	**SinpGlc**	12.8	385				247(50)		223(100)	205(30)
		Disinapoyl derivatives								
			$[M-H]^-$	$MS^2[M-H]^-$					$MS^3[(M-H) \rightarrow (M-H-Sinp)]^-$	
9	**diSinpGentb**	25.3	753	529(100)	223(6)				223(100)	205(80)
10	**diSinpGentb**	26.6	753	529(100)	223(5)				223(100)	205(60)
11	**diSinpGentb**	30.5	753	529(100)	223(4)				223(56)	205(100)
13	**diSinpGlc**	32.4	591	367(100)	223(50)	205(20)				205(100)
		Trisinapoyl derivatives								
			$[M-H]^-$	$MS^2[M-H]^-$		$MS^3[(M-H) \rightarrow (M-H-Sinp)]^-$				
12	**triSinpGentb**	31.9	959	735(100)		529(100)	511(40)	221(30)	205(20)	
		Sinapoyl derivatives from flavonoid glycosides								
			$[M-H]^-$	$MS^2[M-H]^-$		$MS^3[(M-H) \rightarrow (M-H-162)]^-$		$MS^4[(M-H) \rightarrow (M-H-162)) \rightarrow (M-H-162/206)]^-$		
6	**K-3(Sinp)Sophtr-7Glc**	14.3	1139	977(100)		771(100)		609(15)	428(35)	285(100)
				$[M-H-162]^-$		$[M-H-162-206]^-$				
8	**K-3,7diGlc-4′(Sinp)Glc**	24.0	977	815(100)		653(100)				285(100)
				$[M-H-162]^-$		$[M-H-162-162]^-$				

[a] **1**: sinapoylgentiobiose; **2**: 1-sinapoylglucose isomer; **3**: sinapoylgentiobiose isomer; **4**: 1-sinapoylglucose isomer; **5**: 1-sinapoylglucose; **6**: kaempferol-3-(sinapoyl)sophorotrioside-7-glucoside; **8**: kaempferol-3,7-diglucoside-4′-(sinapoyl)glucoside; **9**: 1,2-disinapoylgentiobiose isomer; **10**: 1,2-disinapoylgentiobiose isomer; **11**: 1,2-disinapoylgentiobiose; **12**: 1,2,2′-trisinapoylgentiobiose; **13**: 1,2-disinapoylglucose. **Glc**: Glucoside; **Gentb**: Gentiobioside; **Soph**: Sophoroside; **Sophtr**: Sophorotrioside; **Sinp**: Sinapoyl; **K**: Kaempferol.

2.8.4. Effect on xanthine oxidase activity

The effect of the lyophilized extracts on xanthine oxidase activity was evaluated by measuring the formation of uric acid from xanthine in a double beam spectrophotometer (Heλios α, Unicam), at room temperature, according to a described procedure (Valentão et al., 2001a, 2001 b). The reaction mixtures contained the same proportion of components as in the enzymatic assay for superoxide radical-scavenging activity, except NBT, in a final volume of 750 μl. The absorbance was measured at 295 nm for 2 min. Three experiments were performed in triplicate.

2.9. Hydroxyl radical assay

The deoxyribose method for determining the scavenging effect of the aqueous extracts on hydroxyl radicals was performed according to a described procedure (Valentão et al., 2001 b) in a double beam spectrophotometer (Heλios α, Unicam). Reaction mixtures contained 50 μM ascorbic acid, 40 μM $FeCl_3$, 2 mM EDTA, 2.8 mM H_2O_2, 2.8 mM deoxyribose and lyophilized extracts. All components were dissolved in KH_2PO_4–KOH 10 Mm buffer, pH 7.4. This assay was also performed, either without ascorbic acid or EDTA, in order to evaluate the extracts pro-oxidant and metal chelation potential, respectively. Three experiments were performed in triplicate.

2.10. Hypochlorous acid scavenging activity

The inhibition of hypochlorous acid-induced 5-thio-2-nitrobenzoic acid (TNB) oxidation to 5,5′-dithiobis(2-nitrobenzoic acid) was performed according to a described procedure (Valentão et al., 2001 b), in a double beam spectrophotometer (Heλios α, Unicam). Hypochlorous acid and TNB were prepared immediately before use. Scavenging of hypochlorous acid was ascertained by using lipoic acid as a reference scavenger, which scavenged HOCl in a concentration-dependent manner (data not shown). Three experiments were performed in triplicate.

3. Results and discussion

3.1. Characterization of the seed phenolic compounds

The screening by HPLC-DAD-MS/MS-ESI of the aqueous lyophilized extract of tronchuda cabbage seeds revealed a chromatogram (registered at 330 nm) characterized by the existence of several compounds whose UV spectra had a maximum at ≈330 nm (Fig. 1). Some of them (compounds **6** and **8**) exhibited a second maximum at ≈267 nm, but with lower absorption, indicating the occurrence of flavonoids. These data, together with those obtained in the MS^n Ion-Trap electrospray ionisation fragmentation study, in which can be observed losses of 224/206 amu (sinapic acid/sinapic acid-18) and ions at *m/z* 223/205 (deprotonated sinapic acid/deprotonated sinapic acid-18 ions), indicate that these compounds are sinapic acid derivatives. In addition, the HPLC-DAD chromatographic profile was similar to that found before for *Brassica napus* seeds extracts (Baumert et al., 2005), exhibiting sinapoylcholine, or sinapine (**7**) (UV 325 nm; +MS: 310 $[M]^+$, +MS2$[M]^+$: 251 $[M–(CH_3)_3N]^+$ as an important compound, extensively described to occur in the *Brassica* genus (Bell, 1993; Bouchereau et al., 1991; Shahidi & Naczk, 1992).

Sinapate esters of glucose and gentiobiose (compounds **5**, **9**, **10**, **11**, **12**, **13**) constitute another important group of phenolic compounds already reported in *B. napus* seeds and other plant organs of *B. oleracea* varieties (Ferreres

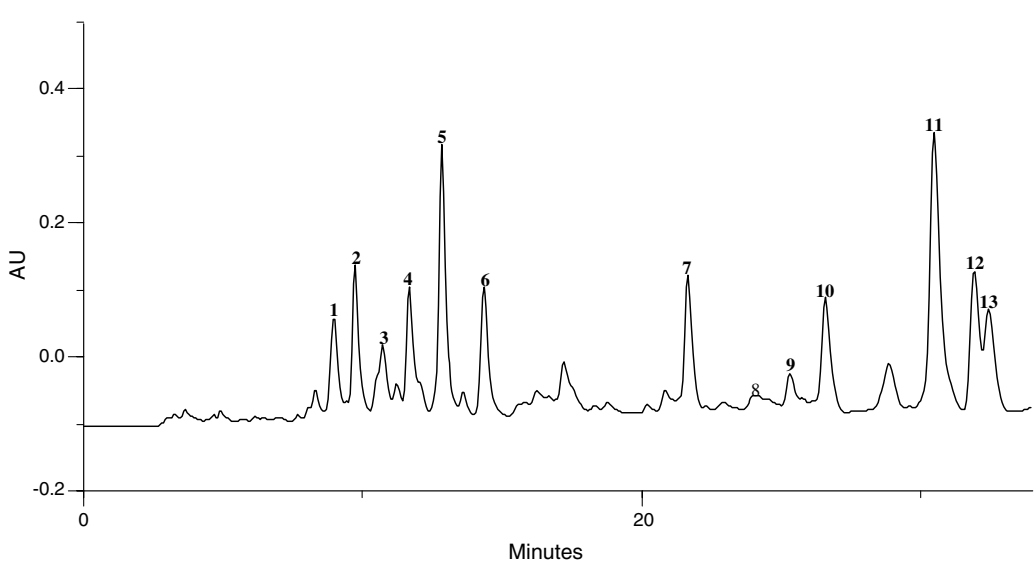

Fig. 1. HPLC-DAD phenolic profile of tronchuda cabbage seeds' aqueous lyophilized extract. Detection at 330 nm. Peaks: (**1**) sinapoylgentiobiose; (**2**) 1-sinapoylglucose isomer; (**3**) sinapoylgentiobiose isomer; (**4**) 1-sinapoylglucose isomer; (**5**) 1-sinapoylglucose; (**6**) kaempferol-3-(sinapoyl)sophorotrioside-7-glucoside; (**7**) sinapoylcholine; (**8**) kaempferol-3,7-diglucoside-4′-(sinapoyl)glucoside; (**9**) 1,2-disinapoylgentiobiose isomer; (**10**) 1,2-disinapoylgentiobiose isomer; (**11**) 1,2-disinapoylgentiobiose; (**12**) 1,2,2′-trisinapoylgentiobiose; (**13**) 1,2-disinapoylglucose.

et al., 2006; Llorach, Gil-Izquierdo, Ferreres, & Tomás-Barberán, 2003; Price, Casuscelli, Colquhoun, & Rhodes, 1997; Vallejo, Tomás-Barberán, & Ferreres, 2004; Vallejo, Tomás-Barberán, & Garcia-Viguera, 2003). The -MS^n fragmentation observed for these compounds was similar to that described in a previous work (Ferreres et al., 2006), mainly consisting in the loss of sinapic acid (224 amu) or sinapic acid-18 (206 amu), giving rise to the base peak in each MS^n event. In the fragmentation of the last sinapic acid-containing ion, there was base peak at *m/z* 223/205 (Table 1). By comparison with data reported before for *Brassica* seeds (Baumert et al., 2005), we tentatively consider that the compounds detected are 1-sinapoylglucose (**5**), 1,2-disinapoylgentiobiose (**11**), 1,2-disinapoylgentiobiose isomers (**9** and **10**), 1,2,2′-trisinapoylgentiobiose (**12**) and 1,2-disinapoylglucose (**13**). Besides these compounds, there were also two 1-sinapoylglucose isomers (compounds **2** and **4**) and two sinapoylgentiobiosides (compounds **1** and **3**), with a MS^n fragmentation distinct from that of the sinapate esters described above. In their $-MS^2[M-H]^-$ the ion at *m/z* 223 is not abundant and the loss of the glycosidic fraction is not complete, forming an ion at *m/z* 247 $[^{0,2}X_0-18]^-$ (Domon & Costello, 1988) as a result of the partial sugar fragmentation (Table 1). The ion $[M-H-18]^-$ is abundant, being the base peak of the sinapoylgentiobiosides **1** and **3**. The observed fragmentation facility to give rise to a total loss of the glycosyl radical in the case of the sinapate esters, or the partial fragmentation of the sugar in the other compounds, suggests that the sugar-sinapic acid link is of a different nature.

In the sinapoyl derivatives from flavonoid glycosides, the compound at R_t 14.3 min formed an ion $[M-H]^-$ whose MS^n fragmentation indicates that its structure is coincident with that of kaempferol-3-(sinapoyl)sophorotrioside-7-glucoside (**6**) (Llorach et al., 2003; Vallejo et al., 2004). Another flavonoid was also detected (compound **8**), presenting a deprotonated molecular ion at *m/z* 977, whose MS^n fragmentation consisted of two sequential losses (MS^2 and MS^3) of glucosyl radical (−162 amu) to form the base peak, and of the loss of glucosyl plus sinapoyl radicals (−162–206) to give rise to a deprotonated kaempferol ion as base peak in MS^4 (Table 1). This kind of fragmentation indicates the occurrence of glycosylation in three phenolic hydroxyls and, according to other authors (Baumert et al., 2005), should be coincident with kaempferol-3,7-diglucoside-4′-(sinapoyl)glucoside.

All the described compounds are reported for the first time in tronchuda cabbage seeds. With the exception of the 1,2-disinapoylgentiobiose isomers and of 1,2,2′-trisinapoylgentiobiose, described in the internal leaves (Ferreres et al., 2006), none of these compounds have been previously reported in tronchuda cabbage.

3.2. Seeds phenolic compounds quantitative analysis

In order to achieve a better characterization of the aqueous lyophilized extract of tronchuda cabbage seeds, its phenolic compounds were quantified by HPLC-DAD. The seeds exhibited a high content of phenolic compounds (≈6.0 g/kg) (Table 2), 1,2-disinapoylgentiobiose (**11**) being the compound present in highest amounts, representing ≈17% of total phenolics, followed by kaempferol-3-(sinapoyl)sophorotrioside-7-glucoside (**6**) and 1-sinapoylglucose (**5**), which corresponded to 15 and 12% of total compounds, respectively. The 1,2-Disinapoylgentiobiose isomer (**9**) was the minor compound, accounting for 2% of total phenolics.

In the phenolic profile of tronchuda cabbage seeds the hydroxycinnamic derivatives are the main phenolics, corresponding to ≈80% of total compounds. This is clearly distinct from what happened with the internal leaves, in which they represented ≈46% of total phenolics (Ferreres et al., 2006), or with the external leaves, in which only flavonol glycosides were determined (Vrchovska et al., 2006).

3.3. Identification and quantification of organic acids by HPLC-UV

Tronchuda cabbage seeds presented a chemical profile composed by seven identified organic acids: aconitic, citric, ascorbic, malic, quinic, shikimic and fumaric acids (Fig. 2). All these compounds have been previously described in tronchuda cabbage leaves (Ferreres et al., 2006; Vrchovska et al., 2006) and are now reported for the first time in its seeds.

The total organic acid content of tronchuda cabbage seeds (≈16 g/kg) (Table 3) was similar to that previously found in the leaves (Ferreres et al., 2006; Vrchovska et al., 2006). However, the seeds exhibited a distinct profile, in which ascorbic acid (**3**) was the main compound, representing ≈52% of total identified organic acids, followed by citric acid (**2**) (≈28% of compounds). As observed with tronchuda cabbage leaves (Ferreres et al., 2006; Vrchovska et al., 2006), shikimic (**6**) and fumaric (**7**) acids were minor

Table 2
Quantification of tronchuda cabbage seed phenolic compounds (mg/kg, dry basis)[a]

	Phenolic compound	Mean	SD
1	Sinapoylgentiobiose	309	0.3
2	1-Sinapoylglucose isomer	368	11.7
3	Sinapoylgentiobiose isomer	270	1.7
4	1-Sinapoylglucose isomer	417	9.8
5	1-Sinapoylglucose	703	11.5
6	Kaempferol-3-(sinapoyl)sophorotrioside-7-glucoside	911	17.7
7	Sinapoylcholine	376	7.1
8	Kaempferol-3,7-diglucoside-4′-(sinapoyl)glucoside	267	17.6
9	1,2-Disinapoylgentiobiose isomer	152	3.2
10	1,2-Disinapoylgentiobiose isomer	345	2.0
11	1,2-Disinapoylgentiobiose	1023	37.1
12	1,2,2′-Trisinapoylgentiobiose	448	2.2
13	1,2-Disinapoylglucose	368	2.4
	$\sum$	5974	

[a] Results are expressed as mean of three determinations. SD standard deviation, $\sum$, sum of the determined phenolic compounds.

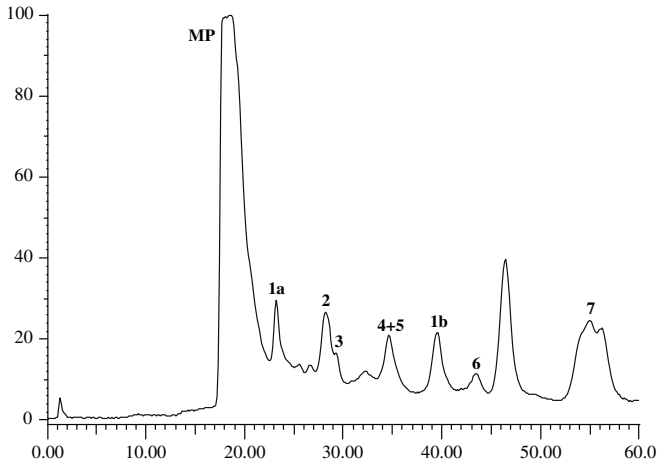

Fig. 2. HPLC-UV organic acid profile of tronchuda cabbage seeds. Detection at 214 nm. Peaks: (**MP**) mobile phase; (**1a** and **1 b**) aconitic acid isomers; (**2**) citric acid; (**3**) ascorbic acid; (**4**) malic acid; (**5**) quinic acid; (**6**) shikimic acid; (**7**) fumaric acid.

Table 3
Quantification of tronchuda cabbage seeds organic acids (mg/kg, dry basis)[a]

	Organic acid	Mean	SD
1a + 1 b	Aconitic	170	2.5
2	Citric	4685	197
3	Ascorbic	8546	438
4 + 5	Malic + quinic	3049	222
6	Shikimic	18.3	0.4
7	Fumaric	39.3	0.5
	$\sum$	16507	

[a] Results are expressed as means of three determinations; SD, standard deviation; $\sum$, sum of the determined organic acids

compounds, accounting for ≈0.1% and 0.2% of total acids, respectively.

3.4. Antioxidant activity

The DPPH· assay provides basic information on the antiradical activity of extracts. In this assay, the scavenging of DPPH· is followed by monitoring the decrease in absorbance at 515 nm, which occurs due to the reduction by the antioxidant (Fukumoto & Mazza, 2000). In the present study the lyophilized extract of tronchuda cabbage seeds displayed a strong concentration-dependent antioxidant potential ($IC_{25} = 64$ μg/ml) (Fig. 3).

Tronchuda cabbage seeds lyophilized extract scavenged X/XO-generated superoxide radical in a concentration-dependent way, as shown in Fig. 4, with an IC_{25} at 197 μg ml^{-1}. A control experiment was performed to determine whether the lyophilized extract might inhibit XO, since an inhibitory effect on the enzyme itself would also lead to a decrease in NBT reduction. (Valentão et al., 2001a). However, it was observed that the lyophilized extract had no effect on XO (Fig. 4). The capacity of the lyophilized extract to scavenge superoxide radical in a concentration-dependent manner was confirmed when this

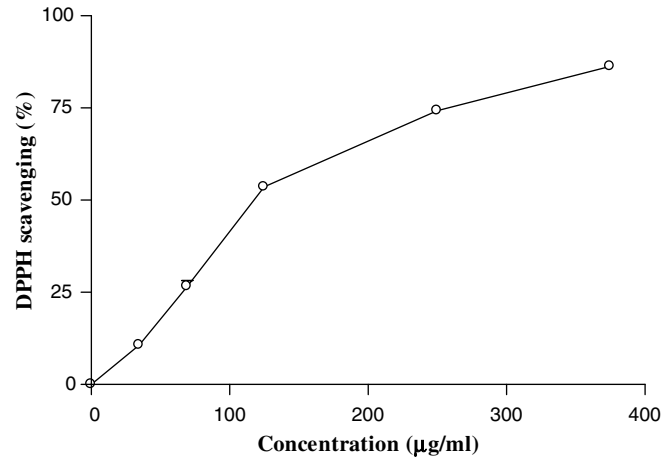

Fig. 3. Effect of tronchuda cabbage seeds on DPPH· reduction. Values show mean ±SE from three experiments performed in triplicate.

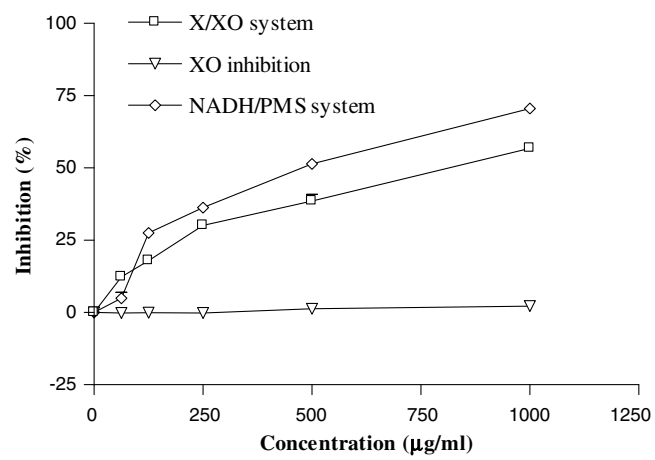

Fig. 4. Effect of tronchuda cabbage seeds against superoxide radical generated in enzymatic (X/XO) and chemical (NADH/PMS) systems and on XO activity. Values show mean ± SE from three experiments performed in triplicate.

radical was generated by a chemical system, which indicated an IC_{25} at 118 μg ml^{-1} (Fig. 4).

The tronchuda cabbage seeds' lyophilized extract also appeared to be a potent scavenger of hydroxyl radical generated by a Fenton system, in a concentration-dependent manner ($IC_{25} = 4$ μg ml^{-1}) (Fig. 5). Some compounds are capable of redox cycling the metal ion required for hydroxyl generation, thus increasing the radical production and, consequently, deoxyribose degradation (Li & Xie, 2000). So, ascorbic acid was omitted in the assay, in order to check the pro-oxidant capacity of the extract. Under those circumstances, tronchuda cabbage seeds' lyophilized extract proved to be an effective substitute of ascorbic acid for concentrations above 1.9 μg ml^{-1} (Fig. 5). This could be attributed to the high amount of this acid in the seeds. It seems that, at the tested concentrations, tronchuda cabbage seeds have both anti-oxidant and pro-oxidant effects, with the first being more pronounced than the latter. Inhibition of iron-dependent deoxyribose degradation in the

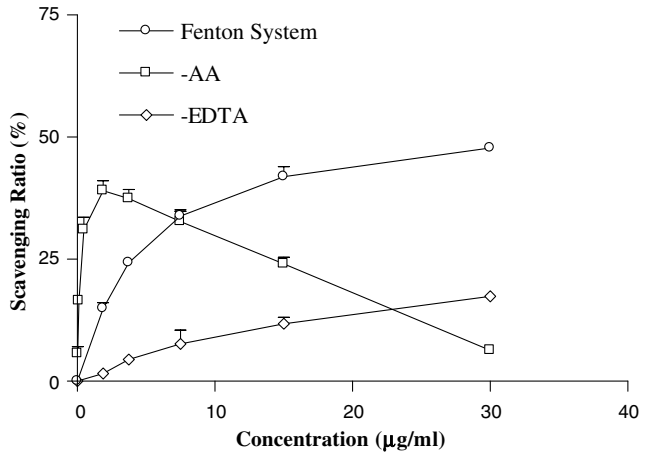

Fig. 5. Tronchuda cabbage seeds' non-specific hydroxyl radical-scavenging activity, pro-oxidant activity (-AA) and specific hydroxyl radical-scavenging (-EDTA). Values show mean ± SE from three experiments performed in triplicate.

absence of EDTA depends not only on the ability of a scavenger to react with hydroxyl radical, but also on its ability to form complexes with iron ions (Halliwell, Gutteridge, & Aruoma, 1987). In the assay performed in the absence of EDTA, tronchuda cabbage seeds' lyophilized extract displayed a concentration-dependent ability to chelate iron ions, with an IC_{10} at 12 μg ml^{-1} (Fig. 5).

Under the present experimental conditions an HOCl scavenger inhibits the oxidation of TNB ($\lambda_{max} = 412$ nm) into DTNB ($\lambda_{max} = 325$ nm) (Künzel, Zee, & Ijzerman, 1996). Tronchuda cabbage seeds' lyophilized extract exhibited a concentration-dependent protective activity against HOCl damage ($IC_{10} = 87$ μg ml^{-1}), as shown in Fig. 6.

According to the results obtained in all assays, and in comparison with data from both tronchuda cabbage internal and external leaves (Ferreres et al., 2006; Vrchovska et al., 2006), it could be concluded that, in general terms, tronchuda cabbage seeds exhibit higher antioxidant potential than do its leaves. This is not surprising, since seeds often contain the highest concentration of lipids of any plant tissue, with high levels of polyunsaturated fatty acids. The occurrence of high amounts of phenolic compounds, particularly of hydroxycinnamic derivatives, and organic acids, namely ascorbic acid, in tronchuda cabbage seeds, suggests that these compounds protect storage lipids from oxidation, as observed with tocopherols (Sattler, Gilliland, Magallanes-Lundback, Pollard, & DellaPenna, 2004), contributing to the viability of seeds and their rapid germination when oxygen demand during germination is high (Andarwulan, Fardiaz, Wattimena, & Shetty, 1999; Randhir & Shetty, 2003; Sattler et al., 2004). In fact, either hydroxycinnamic esters (Plumb, Price, Rhodes, & Williamson, 1997), flavonol glycosides (Braca et al., 2003; Tang, Lou, Wang, Li, & Zhuang, 2001) or organic acids (Silva et al., 2004) are known to exert antioxidant activity.

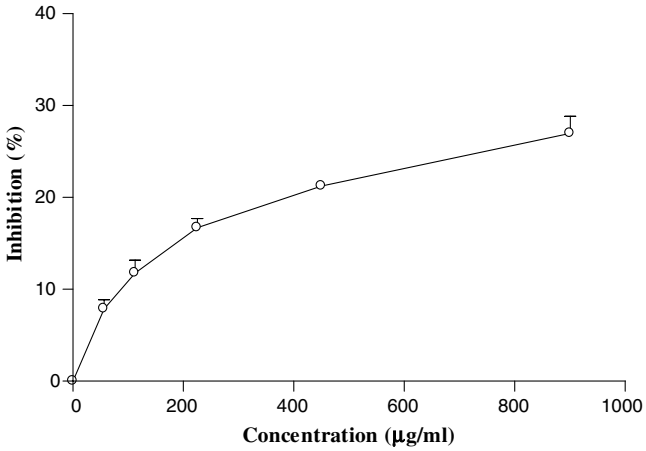

Fig. 6. Effect of tronchuda cabbage seeds on the oxidation of TNB by HOCl. Values show mean ±SE from three experiments performed in triplicate.

In conclusion, the results obtained in this study indicate that tronchuda cabbage seeds may constitute a good source of health-promoting compounds, namely phenolic compounds and organic acids. In addition, the high content of phenolic compounds may be important for the resistance of tronchuda cabbage seeds to downy mildew (Sousa, Dias, & Monteiro, 1997) and insect pests (Ester et al., 2003), as they are known to exert a protective role against parasite attack (Macheix, Fleuriet, & Billot, 1990).

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4.2. Screening of antioxidant compounds during sprouting of *Brassica oleracea* L. var. *costata* DC

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Screening of Antioxidant Compounds During Sprouting of *Brassica oleracea* L. var. *costata* DC

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Abstract: The changes in antioxidant compounds of *Brassica oleracea* L. var. *costata* DC seeds were monitored during the first twelve days of seedling development. Sprouts were screened at time intervals of two days for phenolic compounds and organic acids. The identified phenolic compounds included esters of sinapic acid with glucose, gentiobiose and kaempferol, as well as sinapoylcholine. The organic acids were oxalic, aconitic, citric, pyruvic, malic, shikimic, and fumaric acids. During germination, a depletion of phenolic compounds was observed, although no qualitative changes were seen. Among individual compounds, kaempferol, choline and glucose esters of sinapic acid showed a marked decrease between days two and six, whereas the changes in gentiobiose esters of sinapic acid were smaller. The total organic acids content increased rapidly during the first four days, with less significant variations thereafter. Malic acid, the major organic acid found in sprouts, greatly contributed to this result though oxalic, pyruvic, and fumaric acids also increased in the same manner. In contrast, aconitic, citric and shikimic acids showed decreases between days two and twelve of germination.

Keywords: *Brassica oleracea* L. var. *costata* DC, sprouts, phenolic compounds, organic acids.

1. INTRODUCTION

The leaves and inflorescences of many Brassicaceae vegetables contribute widely to the human diet, especially in winter. Although Brassicaceae seeds can be used for human consumption as an oil (canola seeds) or mixed with some food products (e.g., bread and cake), sprouts, the germinating form of seeds, are favoured for their nutritional value and have became a familiar component in salads [1-3]. Besides, there is considerable interest in the use of *Brassica* sprouts for health benefits. For instance, sprouts of brocolli can be more effective than mature plants for the prevention of carcinogenesis, mutagenesis, and other forms of toxicity of electrophiles and reactive forms of oxygen [4]. Additionally, it has been reported that germination may reduce the content of anti-nutritional components in the seeds, thus making sprouts safe for the diet [5].

Seed germination is a primary step to generate a new plant. In this process triggered by the imbibition of water, the plant embryo resumes growth after a period of quiescence [6]. The germination process implies a series of active and complex biochemical and physiological reactions, resulting in extensive changes in composition and/or morphology [7, 8]. In fact, during embryogenesis, seeds receive primary assimilatory products from the mother plant, such as sugars and amino acids, for the synthesis of the storage products: oil (mainly triacylglycerols) and proteins [9, 10]. From the onset of germination (radicle emergence from the seed coat), this reserve is mobilized in order to support growth during early seedling development [11-14]. The presence of glyoxylate cycle and β-oxidation enzymes, especially abundant during post-germination growth, leads to the breakdown of storage lipids to be used directly in respiration and in the synthesis of carbohydrates [11, 15]. The incorporation of these soluble molecules at the level of citrate in the tricarboxylic acid cycle allows the growing of seedlings until photoautotrophic growth occurs. The tricarboxylic acid cycle also provides intermediates for the biosynthesis of various compounds, including amino acids, purines, pyrimidines, haem, and chlorophyll [14].

Phenolic compounds are also involved in plant development during seed germination. In addition, phenolic compounds can also serve in plant-microbe recognition and signal transduction [16, 17]. The phenolics content of sprouts depends on seed quality and the conditions under which they grow. These include temperature, humidity, and the length of germination [18, 19].

The major phenolic compounds found in *Brassica* seeds are esterified phenolic acids [20]. Phenolic choline esters are characteristic and may contribute to the bitter taste and astringency of seed products. Sinapine, the choline ester of sinapic acid, is the most abundant one in seeds of the Brassicaceae family, being considered a major anti-nutritive com-

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pound [2, 21]. The results obtained with *B. oleracea* seed extract showed that phenolics and organic acids in seeds were important as antioxidants [20]. This antioxidant potential can be especially important during germination, when oxygen demand is high [16]. Based on the antioxidant capacity exhibited by the seeds, we can speculate that the presence of these phenolic compounds and organic acids in sprouts might protect the cell from potential oxidation-induced deterioration, thus making this material a good source of antioxidant compounds for human consumption.

The Brassicaceae *Arabidopsis thaliana*, frequently used as a model system in genetics and plant development works, has been studied for regulation of flavonoid gene expression [22, 23] and lipid breakdown [15, 24] in seeds and germinating seedlings. Previous work with *B. oleracea* sprouts concerned the genetic characterization of seed germination [25, 26], monitorization of cell cycle modifications during the first divisions of the seeds [6], sensitivity to hypoxia [18], and the ability of seeds produced under a drought to germinate normally [27]. Other studies have dealt with the ability of seeds to biosynthesize and incorporate erucic acid into triacylglycerols [28] and the activity and distribution of peroxidases isozymes in germinating seeds [29]. The determination of isothiocyanate and glucosinolate content in 3-day-old sprouts of broccoli, and the study of its effects as inducers of enzymes of xenobiotic metabolism in murine hepatoma cells was also carried out [4].

This work is an attempt to establish the relationships among primary and secondary metabolism occurring during the developmental processes of young sprouts, especially in what concerns the changes in organic acids and phenolic compounds. For this purpose, phenolic compounds and organic acids were screened by HPLC/DAD and HPLC/UV, respectively, for a twelve days germination period.

2. MATERIALS AND METHODS

2.1. Standards and Reagents

Oxalic, malic, shikimic and fumaric acids were purchased from Sigma (St. Louis, MO, USA). Aconitic, citric, pyruvic, ascorbic and sinapic acids and kaempferol-3-*O*-rutinoside were from Extrasynthése (Genay, France). Hide powder was obtained from FILK (Freiberg, Germany). Methanol and acetic acid were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Seeds Germination

Brassica oleracea L. *var. costata* DC seeds were collected in 2005 in Bragança, Northeast Portugal. Two hundred seeds were placed on 15 cm diameter Petri dishes lined with fiberglass and watered with 200 mL of distilled water to maintain approximately 100% relative humidity throughout the germination period. The seeds were germinated at 20-23.5ºC, under a 16 h-light/8 h-dark regime. A seed sample was used as control. Every 2 days, 5 plates were withdrawn, and germination counts and sprout weight were registered until twelve days of germination. For day 8 only 4 plates and for day 12 just 1 plate were considered.

The harvested sprouts were freeze-dried (Labconco 4.5 Freezone apparatus, Kansas City, MO, USA), ground and stored in a dessicator. Changes of moisture and dry solid during germination were determined after freeze-drying.

2.3. Sample Preparation

An aqueous extract was prepared as follows: ~0.5 g of the freeze-dried sprouts were boiled for 15 min in 200 mL of water and then filtered using a Büchner funnel. The resulting extract was lyophilized and kept in a dessicator in the dark. For the determination of phenolic compounds and organic acids, the lyophilized extract was redissolved in water and in sulfuric acid 0.01 M, respectively, and filtered (0.45 µm).

In order to assess possible interferences in the HPLC analysis of phenolic compounds, an analysis of tannins was performed, by treatment with hide powder: 250 µL of redissolved aqueous extract (50 mg/mL) were thoroughly mixed with 2.5 mg hide powder for 1 h and then filtered (0.45 µm).

2.4. HPLC-DAD Analysis of Phenolic Compounds

The analysis of phenolic compounds was carried out as reported previously [20] using HPLC (Gilson) and a RP-18 LiChroCART (Merck, Darmstadt, Germany) column (250 x 4 mm, 5 µm particle size), protected with a 4 x 4 mm LiChroCART guard column. Elution was performed using acetic acid 1% (A) and methanol (B) as solvents, starting with 20% B and using a gradient to obtain 50% B at 30 min and 80% B at 37 min. The flow rate was 1 mL/min, and the injection volume was 20 µL. UV spectra were acquired over the range 200-400 nm using a Gilson diode array detector, and chromatograms were recorded at 320 nm. The data were processed on Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities. The quantification of phenolic compounds was based on the absorbance in the chromatograms relative to external standards. Since standards of the compounds were not commercially available, sinapic acid derivatives were quantified as sinapic acid and the kaempferol derivatives as kaempferol-3-*O*-rutinoside.

2.5. HPLC-UV Analysis of Organic Acids

The separation of the organic acids was carried out as previously reported [30], in a system consisted of an analytical HPLC unit (Gilson) with an ion exclusion column, Nucleogel® Ion 300 OA (300 × 7.7 mm) (Macherey-Nagel, Düren, Germany) in conjunction with a column heating device set at 30°C. Briefly, elution was carried out isocratically at a solvent flow rate of 0.2 mL/min, with 0.01 M sulfuric acid. UV detection was used at 214 nm. Organic acids quantification was based on the absorbance recorded in the chromatograms relative to external standards.

2.6. Data Analysis

For organic acids and phenolic compounds quantitation, triplicate experiments were conducted, and the results are presented as mean ± standard deviation. ANOVA followed by Newman-Keuls test ($p < 0.05$) was used to analyse the differences between germination days for phenolic compounds and organic acids.

3. RESULTS AND DISCUSSION

3.1. Seed Viability and Dry Matter Losses During Sprouting

The environment during seed production can have a significant effect on seed performance [18]. Seeds must have 40-60% moisture content for germination to occur and begin to germinate after 24 h of imbibition [29]. On day 2, the seeds had an average moisture content of 66.0%, and 68.3% were already germinated (Table **1**). Seed viability was generally high, reaching 81.0% after 4 days of germination. The loss of viability after this period can be attributed to the fact that not all of the germinated seeds were successful in seedling establishment. Over the 12 days development, the sprouts fresh weight increased in a linear manner, from an average of 9.0 mg at day 2 to almost 56 mg by day 12 (Table **1**).

Table 1. Changes in Sprout Viability, Fresh Weight and Moisture Content with Germination Time

Germination Time (Days)	Seed Viability (%)	Sprout Fresh Weight (mg)	Moisture Content %
2	68.3±8.52	9.0±0.16	66.0±1.84
4	81.0±6.28	15.6±1.12	79.2±1.61
6	76.6±6.40	23.7±2.37	84.0±1.30
8	71.0±8.57	44.1±6.05	86.7±0.68
10	68.9±4.25	48.8±2.76	89.8 ±2.07
12	58.7	55.7	91.1

Results are expressed as the means of germination percentages obtained in 5 replicates ± standard error.

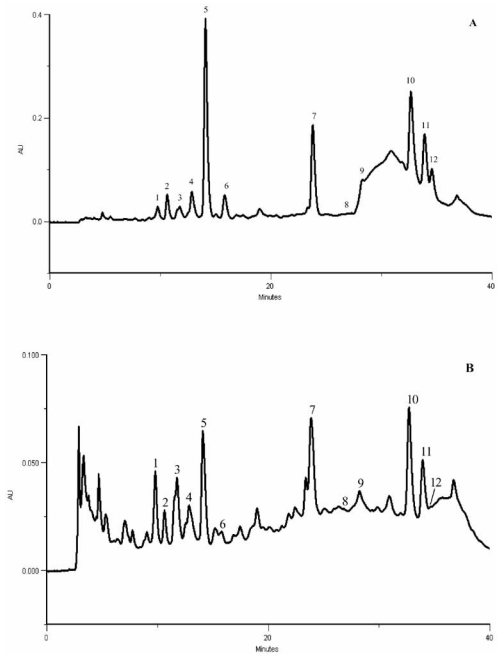

Fig. (1). HPLC-DAD phenolic profile of *B. oleracea* sprouts aqueous lyophilized extract. Detection at 320 nm. **A**) 2 days germination; **B**) 12 days germination. Peaks: (**1**) sinapoylgentiobiose; (**2**) 1-sinapoylglucose isomer; (**3**) sinapoylgentiobiose isomer; (**4**) 1-sinapoylglucose isomer; (**5**) 1-sinapoylglucose; (**6**) kaempferol-3-(sinapoyl)sophorotrioside-7-glucoside; (**7**) sinapoylcholine; (**8**) 1,2-disinapoylgentiobiose isomer; (**9**) 1,2-disinapoylgentiobiose isomer; (**10**) 1,2-disinapoylgentiobiose; (**11**) 1,2,2'-trisinapoylgentiobiose; (**12**) 1,2-disinapoylglucose.

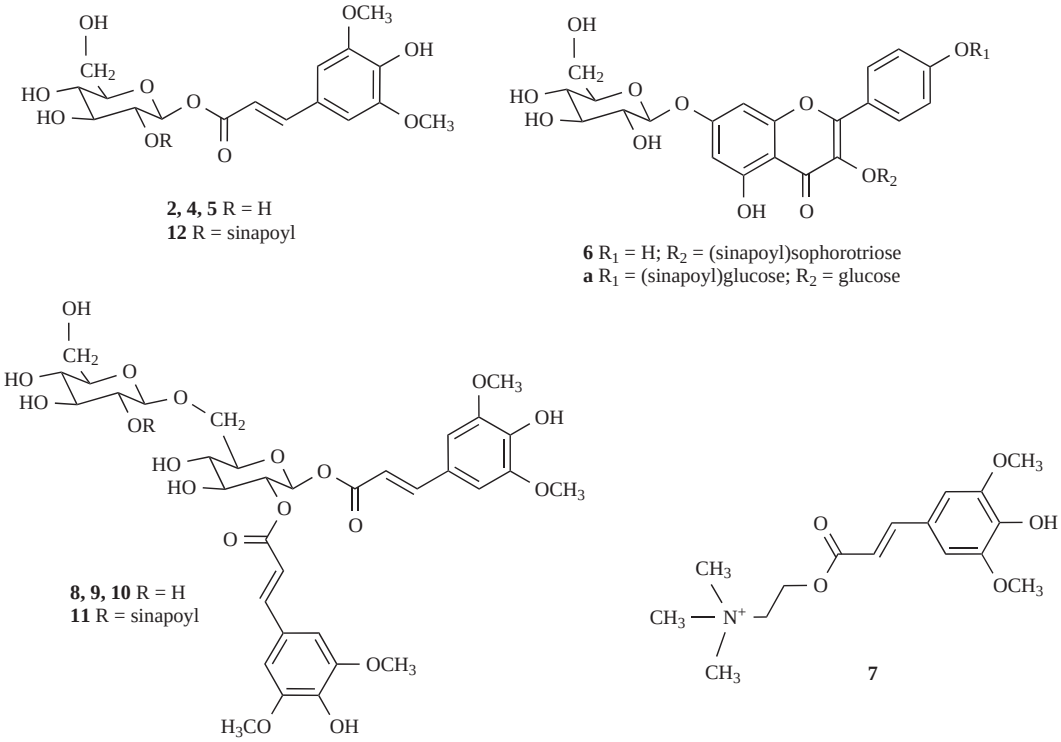

Fig. (2). Chemical structures of phenolic compounds quantified in *B. oleracea* sprouts. (**2**) 1-sinapoylglucose isomer; (**4**) 1-sinapoylglucose isomer; (**5**) 1-sinapoylglucose; (**6**) kaempferol-3-(sinapoyl)sophorotrioside-7-glucoside; (**7**) sinapoylcholine; (**8**) 1,2-disinapoylgentiobiose isomer; (**9**) 1,2-disinapoylgentiobiose isomer; (**10**) 1,2-disinapoylgentiobiose; (**11**) 1,2,2'-trisinapoylgentiobiose; (**12**) 1,2-disinapoylglucose; (**a**) kaempferol 3,7-diglucoside-4'-(sinapoyl)-glucoside.

3.2. Changes in Phenolic Compounds During Sprouting

The phenolics composition of *B. oleracea* L. var. *costata* sprouts was monitored during 12 days of germination. The HLPC/DAD analysis showed the presence of 12 phenolic compounds already identified in the seeds of *B. oleracea*, at all germination times [20]. These compounds include five sinapoyl glucosides, four disinapoyl glucosides, one tris-inapoyl glucoside, sinapoyl choline, and one kaempferol glucoside acylated with sinapic acid (Figs. **1**,**2**).

In the chromatogram of sprouts with 2 days of germination (Fig. **1A**), a wide hump is present ~30 min, which is characteristic of tannins. The sample treatment with hide powder was not able to remove this, indicating that tannins were not the responsible for the hump.

As it was previously shown for the seeds [20], phenolic acids derivatives were the predominant phenolics found in the sprouts of *B. oleracea* (Table **2**). The total phenolics content of sprouts between 2 and 12 days of germination showed significant quantitative changes (Fig. **3**): the phenolic compounds were depleted throughout the germination period, having a marked decrease between days 2 and 6. The total amount decreased 85%, from ~11.1 g/kg in sprouts with 2 days to ~1.6 mg/kg in sprouts with 12 days of germination. The decrease in phenolic compounds can be explained by its utilisation for cell wall biosynthesis and as antioxidants [16-17, 23]. Some losses in phenolics can also be explained by the leaching of the water-soluble free phenolic acids upon the imbibition of dry seeds [19].

A seed sample was analysed along with the sprouts to serve as a control. Even though this sample was previously characterised [20], seed quality continues to increase after physiological maturity, with possible changes in phenolic composition during storage time [7, 27]. The amount of phenolic compounds in seeds was ~9.0 g/kg (Table **2**), which included 489 mg/kg of kaempferol 3,7-diglucoside-4'-(sinapoyl)-glucoside, a compound with a retention time of 28.5 min, not detected in the sprouts (data not shown). This amount is lower than the average amount found in sprouts with 2 days of germination, although the difference is not significant (Fig. **3**).

In what concerns the individual compounds, the glucose esters of sinapic acid showed a significant decrease during the germination period, especially relevant between days 2 and 6 of germination (Fig. **4**). In fact, until the 12th day of germination 1-sinapoylglucose isomer (**2**), 1-sinapoylglucose isomer (**4**), 1-sinapoylglucose (**5**) and 1,2-disinapoylglucose (**12**) decreased between 76 and 98% of their initial content. For kaempferol-3-(sinapoyl)sophorotrioside-7-glucoside (**6**) and sinapoylcholine (**7**) the reduction observed corresponded to 96 and 85% respectively.

The decrease of sinapoylcholine (**7**) in sprouts from 1.7 g/kg on day 2 to 0.3 g/kg on day 12 is a positive feature of the germination process once this compound is considered anti-nutritive [21]. This decrease in sinapoylcholine content can be ascribed to the fact that during the germination of seedlings, sinapoylcholine is hydrolysed to choline, an important substrate in the methylation cycle, and sinapic acid. As a consequence, an increase in sinapic acid would be expected through the germination. However, free phenolic acids can be polymerized for lignification and structure development on the cell walls of growing seedlings, which may explain the absence of sinapic acid.

Some gentiobiose esters of sinapic acid also decreased significantly, although these changes occurred more gradually within the germination time. Nevertheless, the depletion noticed for compounds **8** to **11**, varied between 82 and 92% of the initial amount. Except for compound **8**, the decrease of the gentiobiose esters of sinapic acid was <60% on day 10, which might indicate that the germinating seeds preferably use the glucose esters of sinapic acid during germination. The sinapoylgentiobiose isomers (**1**) and (**3**) displayed the smallest variations, decreasing less than 25% until the 12th day of germination.

In terms of relative amounts, the phenolic profile of the sprouts showed the presence of 4 major compounds at all of the analysed germination times: 1-sinapoylglucose (**5**), sinapoylcholine (**7**), 1,2-disinapoylgentiobiose (**10**) and 1,2,2'-trisinapoylgentiobiose (**11**). These compounds accounted for 73 and 68% of total phenolics between days 2 and 12.

The relative amounts of 1-sinapoylglucose isomer (**2**) (~4%), 1-sinapoylglucose isomer (**4**) (~6%), 1,2-disinapoylgentiobiose isomer (**9**) (~2%) and sinapoylgentiobiose isomer (**3**) (~2%) did not change significantly throughout the germination period, but, sinapoylgentiobiose (**1**) increased from ~1.4% to 7.4%. On the other hand, kaempferol-3-(sinapoyl)-sophorotrioside-7-glucoside (**6**) and 1,2-disinapoylglucose (**12**) decreased from ~7.5% to 2.2% and ~6.6% to 0.7%, respectively. The 2-disinapoylgentio-biose isomer (**8**) was a minor compound, representing <0.5% of total phenolics.

3.3. Changes in Organic Acids During Sprouting

The screening of organic acids belonging to glycolysis, tricarboxylic acid and glyoxylate cycles showed the presence of oxalic, aconitic, citric, pyruvic, malic, shiquimic, and fumaric acids (Figs. **5**,**6**). These acids were previously described in *B. oleracea* leaves and seeds [20, 21, 31], with the exception of pyruvic acid that is now reported for the first time in its sprouts. The total organic acids content increased ~46% from 45.7 g/kg after 2 days of germination to 66.9 g/kg on day 12 (Table **3** and Fig. **7**). This may be explained by the increased metabolic activity of the seeds, which rapidly resume the glycolytic and the tricarboxylic acid cycle and the β-oxidation of fatty acids after germination [11, 15].

Seeds had an amount of organic acids lower than that present in the sprouts. Ascorbic acid (retention time of 34.2 min), one of the major compounds found in the seeds (~13.3 g/kg, data not shown) was present only in vestigial amounts in the sprouts, being greatly depleted since the beginning of germination, may be due to its well known strong antioxidant effect.

Citric (**3**) and malic (**5**) acids were the major organic acids found in sprouts at all germination times (Table **3**). Citric acid, accounting for more than 15% of the total organic acids content, decreased ~37% from day 2 to day 12 (Fig. **8**). However, despite the significant decrease between days 2 and 4 and increase between days 6 and 8, its variation during

Table 2. Changes in Phenolic Compounds of *B. oleracea* Sprouts with Germination Time (mg/kg, Dry Basis)[a]

Compound	Seed	2 Days	4 Days	6 Days	8 Days	10 Days	12 Days
1 Sinapoylgentiobiose	240.1 ± 0.44	132.4 ± 12.89 214.1 ± 6.81 158.3 ± 1.11 193.9 ± 0.19 91.4 ± 2.19	189.5 ± 1.52 159.5 ± 10.45 213.7 ± 14.16 186.6 ± 7.85 209.5 ± 4.66	187.7 ± 3.53 144.8 ± 0.74 145.4 ± 3.00 233.5 ± 2.03 140.0 ± 0.40	143.3 ± 3.85 139.4 ± 2.44 187.5 ± 0.04 151.2 ± 9.32	177.2 ± 6.74 143.9 ± 3.24 133.7 ± 2.65 181.3 ± 10.35 161.3 ± 2.09	120.3 ± 6.50
2 1-Sinapoylglucose isomer	381.9 ± 37.00	302.9 ± 3.26 454.3 ± 8.78 317.4 ± 4.40 326.9 ± 22.05 204.3 ± 5.77	288.3 ± 0.19 263.0 ± 1.33 369.5 ± 11.04 304.5 ± 3.31 369.5 ± 2.92	144.4 ± 2.39 99.6 ± 2.45 100.1 ± 1.64 189.3 ± 5.38 117.1 ± 1.28	111.1 ± 0.33 120.8 ± 4.00 188.5 ± 0.63 138.9 ± 10.12	83.8 ± 4.82 72.3 ± 0.51 48.2 ± 1.72 103.6 ± 0.79 78.1 ± 5.51	58.9 ± 0.45
3 Sinapoylgentiobiose isomer	206.6 ± 23.38	203.6 ± 2.95 280.6 ± 1.47 201.7 ± 0.61 243.0 ± 2.44 119.5 ± 1.54	167.5 ± 1.09 155.0 ± 0.65 174.7 ± 12.08 178.4 ± 27.77 202.8 ± 0.13	221.7 ± 7.02 175.7 ± 3.08 177.0 ± 0.69 194.7 ± 4.35 182.8 ± 3.01	193.6 ± 1.91 173.8 ± 7.05 220.4 ± 3.18 196.4 ± 29.84	180.6 ± 6.80 213.9 ± 15.66 156.3 ± 5.02 232.5 ± 1.13 195.8 ± 9.09	163.3 ± 2.66
4 1-Sinapoylglucose isomer	551.0 ± 19.96	360.2 ± 3.46 476.9 ± 2.91 444.5 ± 1.67 469.5 ± 18.2 269.3 ± 0.58	340.7 ± 0.74 284.5 ± 0.76 355.6 ± 34.45 348.8 ± 5.70 365.2 ± 18.60	184.3 ± 6.47 133.3 ± 8.00 146.9 ± 0.20 209.3 ± 14.71 145.7 ± 4.74	145.3 ± 1.27 200.7 ± 5.15 280.7 ± 6.10 194.9 ± 15.01	144.6 ± 4.95 63.7 ± 3.34 57.5 ± 0.76 97.5 ± 6.68 105.3 ± 6.37	98.3 ± 1.32
5 1-Sinapoylglucose	1320.1 ± 25.34	2810.2 ± 28.73 3505.6 ± 21.79 3036.9 ± 31.88 2779.1 ± 156.34 1254.0 ± 31.38	1614.5 ± 22.28 1596.9 ± 68.77 1946.0 ± 35.29 1684.9 ± 8.87 2020.3 ± 37.21	796.6 ± 20.89 630.9 ± 12.70 581.4 ± 9.35 1168.1 ± 16.39 540.4 ± 0.78	533.1 ± 1.93 575.3 ± 2.55 745.8 ± 6.17 536.3 ± 26.45	210.4 ± 9.86 293.4 ± 8.36 234.7 ± 6.48 333.8 ± 18.59 342.9 ± 9.53	241.5 ± 4.07
6 Kaempferol-3-(sinapoyl)sophorotrioside-7-glucoside	795.9 ± 13.49	828.4 ± 0.70 990.3 ± 11.98 850.2 ± 11.27 933.7 ± 21.97 544.9 ± 7.97	365.5 ± 1.72 337.9 ± 22.54 424.2 ± 20.89 403.0 ± 11.41 433.5 ± 21.54	119.0 ± 4.23 143.6 ± 1.18 100.6 ± 1.77 180.0 ± 2.96 81.6 ± 2.96	152.4 ± 18.31 239.4 ± 0.80 291.5 ± 17.51 193.6 ± 3.49	103.0 ± 7.23 159.2 ± 14.51 143.1 ± 4.41 167.0 ± 3.40 190.0 ± 21.55	35.9 ± 0.46
7 Sinapoylcholine	2064.9 ± 14.55	1608.8 ± 8.91 2606.6 ± 83.56 2356.1 ± 17.53 2606.6 ± 83.56 2408.0 ± 45.12	1535.6 ± 4.53 1482.3 ± 74.67 1882.8 ± 84.71 1720.1 ± 5.59 1811.3 ± 52.66	939.3 ± 84.67 813.7 ± 8.11 790.7 ± 71.64 1074.4 ± 57.36 601.1 ± 42.12	594.4 ± 11.28 764.3 ± 28.17 819.0 ± 19.50 701.6 ± 9.43	322.5 ± 16.04 436.1 ± 5.35 337.1 ± 16.45 375.1 ± 21.02 311.7 ± 0.81	348.5 ± 35.59
8 1,2-Disinapoylgentiobiose isomer	179.6 ± 0.23	48.4 ± 0.12 39.5 ± 0.62 44.0 ± 0.97 42.9 ± 1.57 nq	18.5 ± 0.42 17.3 ± 1.03 23.7 ± 3.41 16.2 ± 4.35 23.0 ± 0.16	4.6 ± 0.55 7.3 ± 0.32 7.1 ± 0.17 4.3 ± 0.14 3.7 ± 0.21	13.6 ± 0.15 12.2 ± 0.40 15.6 ± 0.34 14.2 ± 1.63	11.8 ± 0.11 11.9 ± 0.25 9.7 ± 0.12 9.5 ± 0.83 15.1 ± 0.77	3.7 ± 0.36
9 1,2-Disinapoylgentiobiose isomer	150.5 ± 16.34	229.6 ± 5.30 265.5 ± 9.51 401.5 ± 29.81 266.0 ± 18.44 181.9 ± 5.43	192.1 ± 16.61 93.2 ± 5.15 204.3 ± 9.71 175.8 ± 9.39 181.4 ± 3.83	128.3 ± 4.34 98.5 ± 4.93 49.0 ± 6.08 196.8 ± 1.52 107.0 ± 9.87	76.8 ± 0.09 85.0 ± 1.42 117.9 ± 4.64 165.6 ± 19.40	132.0 ± 1.88 225.5 ± 31.70 110.9 ± 7.08 139.4 ± 9.59 183.0 ± 10.33	30.7 ± 1.06
10 1,2-Disinapoylgentiobiose	1410.7 ± 28.53	1630.5 ± 6.77 2527.7 ± 21.54 2033.7 ± 2.05 2119.8 ± 103.01 884.3 ± 18.12	1259.3 ± 77.14 1078.9 ± 27.46 1514.3 ± 80.47 1584.9 ± 32.08 1685.8 ± 76.75	1152.1 ± 34.16 970.6 ± 15.62 739.1 ± 11.80 1643.8 ± 22.63 851.1 ± 4.74	760.9 ± 27.22 930.7 ± 27.34 1172.0 ± 18.87 847.1 ± 30.48	657.9 ± 13.12 897.2 ± 70.61 587.1 ± 15.04 835.3 ± 29.59 740.5 ± 4.03	329.8 ± 4.21
11 1,2,2'-Trisinapoylgentiobiose	635.4 ± 36.33	1233.1 ± 1.49 1541.2 ± 67.19 1427.6 ± 5.15 1553.3 ± 91.42 710.5 ± 22.09	1062.4 ± 7.50 1013.3 ± 17.34 1269.2 ± 20.37 1331.7 ± 2.47 1427.2 ± 78.57	865.8 ± 32.27 795.6 ± 9.11 587.7 ± 13.95 1361.6 ± 46.43 692.3 ± 4.73	640.7 ± 26.63 700.7 ± 6.87 910.0 ± 19.67 645.2 ± 31.20	570.0 ± 11.23 805.0 ± 26.35 470.6 ± 15.94 594.8 ± 11.47 639.6 ± 17.96	181.3 ± 7.74
12 1,2-Disinapoylglucose	566.7 ± 24.67	585.1 ± 15.92 845.2 ± 10.72 815.3 ± 1.57 791.2 ± 46.72 617.8 ± 0.70	299.1 ± 30.05 296.4 ± 31.59 452.2 ± 6.83 450.8 ± 44.87 455.5 ± 17.47	199.0 ± 5.92 165.9 ± 5.11 117.4 ± 1.32 347.2 ± 4.33 137.9 ± 6.80	99.6 ± 1.63 154.5 ± 9.35 172.4 ± 1.72 165.8 ± 27.47	46.3 ± 1.08 56.3 ± 0.76 38.6 ± 1.07 57.1 ± 3.45 51.8 ± 0.98	181.3 ± 7.74
Total	8503.4	9973.2 13747.4 12087.2 12325.7 7286.0	7332.9 6778.3 8830.2 8386.0 9184.9	4942.9 4179.5 3542.4 6803.1 3600.6	3428.8 4096.8 5121.2 3950.7	2640.2 3378.5 2327.4 3127.0 3015.1	1623.9

[a]Results are expressed as mean of three determinations± standard deviation.

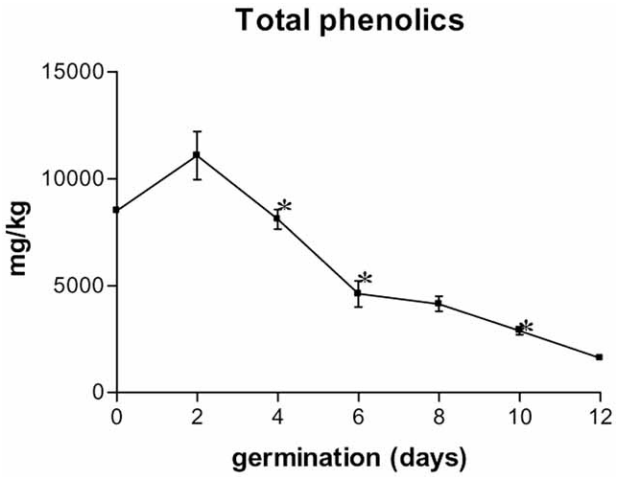

Fig. (3). Evolution in total phenolic compounds content of *B. oleracea* sprouts with germination time. * p<0.05, compared with the previous germination time.

the germination period did not show a clear tendency. Malic acid increased from 27.9 g/kg (61% of the total organic acids) on day 2 to 51.6 g/kg (77% of the total organic acids) on day 12, which represents an increase of 85% along the germination period. This was the organic acid that registered the highest increase, which might indicate that besides β–oxidation, the glyoxylate cycle in which fatty acids are converted to sugars having malate as an intermediate product, was active [14].

Pyruvic acid (**4**), which was not detected in seeds and still not quantifiable in sprouts with 2 days, represented ~4% of the total organic acids thereafter. This may be due to its production during glycolysis, as a consequence of the increased respiration rate [13]. The overall amount of oxalic acid (**1**) did not change significantly. This acid represents less than 4% of the total organic acids at all germination times. Aconitic acid (**2**), which represented ~0.8% of the total amount of organic acids in seeds, was found to be the minor compound in the sprouts (~0.1%).

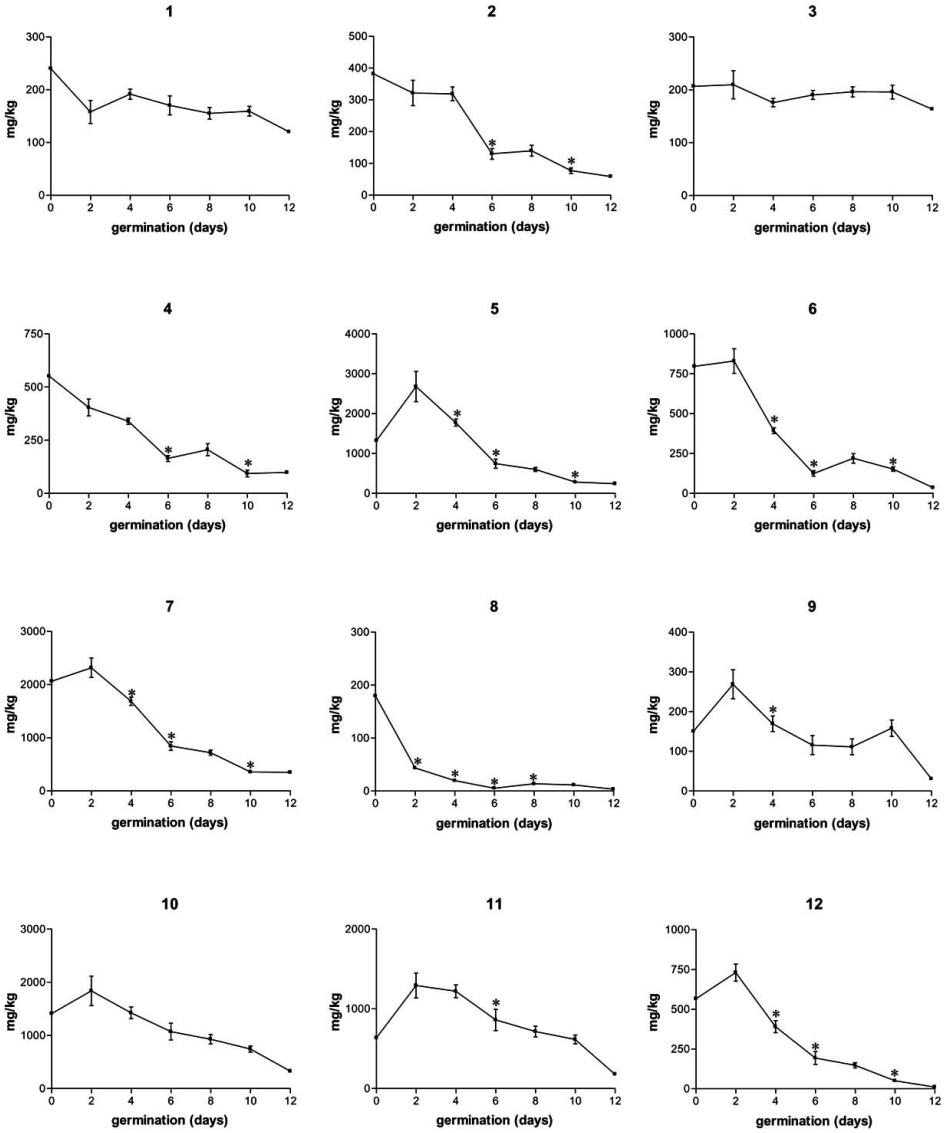

Fig. (4). Changes in individual phenolic compounds of *B. oleracea* sprouts with germination time. Numbers of panels refer to the number of compounds (see caption of Fig. **1**). * p<0.05, compared with the previous germination time.

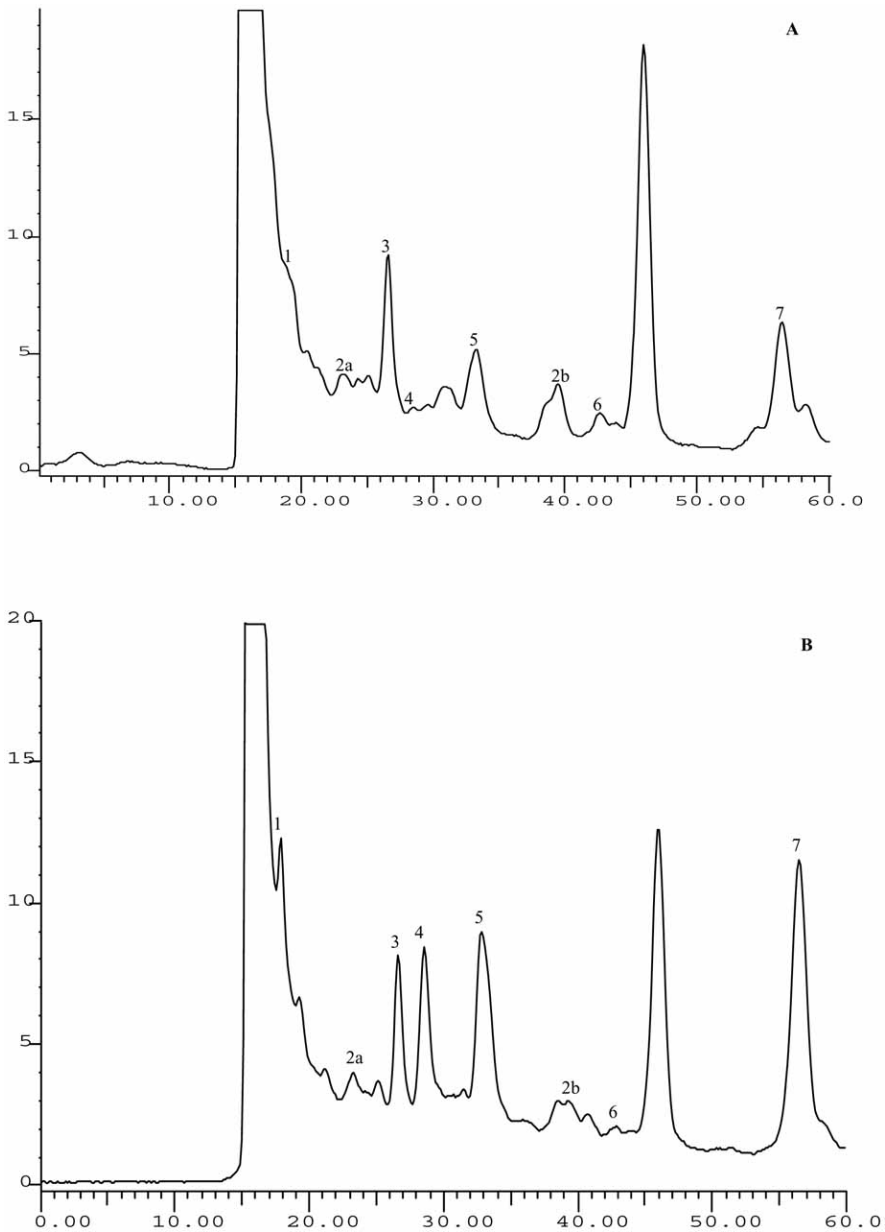

Fig. (5). HPLC-UV organic acid profile of *B. oleracea* sprouts. Detection at 214 nm. **A**) 2 days germination; **B**) 12 days germination. Peaks: (**1**) oxalic acid; (**2a** and **2b**) aconitic acid isomers; (**3**) citric acid; (**4**) pyruvic acid; (**5**) malic acid; (**6**) shikimic acid; (**7**) fumaric acid.

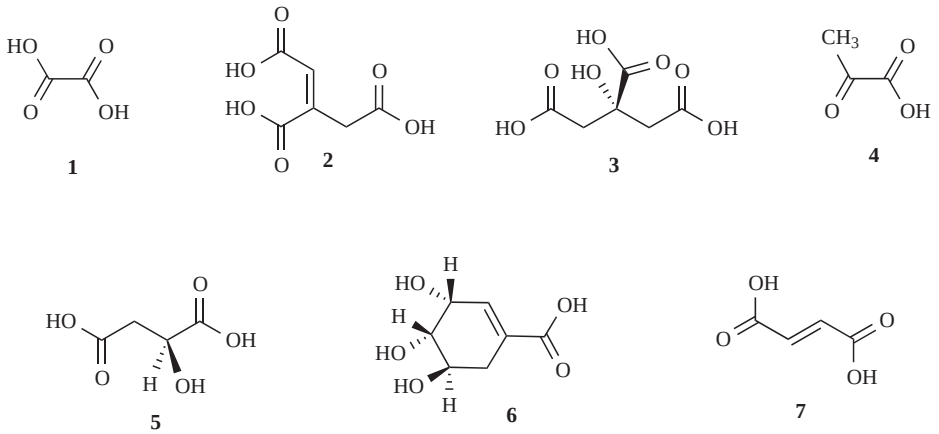

Fig. (6). Chemical structures of organic acids quantified in *B. oleracea* sprouts. (**1**) oxalic acid; (**2a** and **2b**) aconitic acid isomers; (**3**) citric acid; (**4**) pyruvic acid; (**5**) malic acid; (**6**) shikimic acid; (**7**) fumaric acid.

Table 3. Changes in Organic Acids of *B. oleracea* Sprouts with Germination Time (mg/kg, Dry Basis)[a]

Organic Acid	Seed	2 Days	4 Days	6 Days	8 Days	10 Days	12 Days
1 Oxalic	821.7 ± 112.31	nq nq nq nq nq	950.5 ± 49.3 1248.7 ± 96.23 1740.8 ± 28.31 1266.1 ± 7.89 1724.2 ± 241.64	1392.9 ± 29.95 748.6 ± 5.31 1419.8 ± 42.44 729.9 ± 0.66 668.4 ± 4.36	2197.1 ± 74.26 1946.0 ± 17.02 1122.4 ± 49.19 2597.2 ± 17.31	862.0 ± 19.49 2523.2 ± 38.71 1960.5 ± 37.94 2202.2 ± 150.89 1204.9 ± 25.71	1094.4 ± 104.12
2 Aconitic	275.8 ± 6.73	39.0 ± 2.86 25.0 ± 3.09 13.6 ± 1.63 15.4 ± 0.45 34.5 ± 3.13	89.4 ± 3.5 50.7 ± 10.44 96.5 ± 12.84 47.5 ± 0.18 122.6 ± 10.40	43.5 ± 2.80 26.8 ± 0.72 18.4 ± 0.12 31.7 ± 0.31 45.6 ± 0.85	41.8 ± 0.94 27.9 ± 2.81 120.6 ± 0.37 23.6 ± 5.42	68.3 ± 1.18 27.9 ± 1.22 30.1 ± 2.67 34.2 ± 5.68 32.4 ± 4.32	37.8 ± 1.53
3 Citric	13334.1 ± 849.9	15686 ± 1622.7 18435 ± 130.5 17085 ± 6.1 17807 ± 24.4 15513 ± 1069.5	9966.5 ± 27.0 11882.4 ± 1311.2 12247.4 ± 403.57 13138.8 ± 311.63 15118.9 ± 922.26	9456.0 ± 682.86 15048.5 ± 443.56 13497.2 ± 91.06 14912.5 ± 447.32 9279.7 ± 54.54	14096.6 ± 624.23 16303.3 ± 79.39 23261.5 ± 180.98 20250.8 ± 367.10	14034.1 ± 344.83 21588.4 ± 101.14 13058.5 ± 178.14 16137.9 ± 1167.13 16033.1 ± 103.41	10583.2 ± 553.54
4 Pyruvic	nq	nq nq nq nq nq	1750.2 ± 21.8 1748.1 ± 32.39 2220.3 ± 62.89 1999.6 ± 98.48 2340.5 ± 245.72	2923.1 ± 109.62 2487.8 ± 83.28 3156.0 ± 242.45 1946.8 ± 58.40 2334.6 ± 174.25	1984.8 ± 90.52 1752.3 ± 3.75 2316.4 ± 34.63 2624.0 ± 33.33	2782.9 ± 112.33 3191.5 ± 278.86 2888.2 ± 145.11 2908.3 ± 286.89 1846.5 ± 49.11	2637.5 ± 65.03
5 Malic	19599.2 ± 289.57	29282.8 ± 2227.04 24967.2 ± 77.47 30442.3 ± 595.07 24422.5 ± 64.18 30454.2 ± 107.24	53854 ± 256.4 56123 ± 771.2 47944 ± 2280.8 59205 ± 2593.3 58901± 981.0	51619 ± 2174.6 38951 ± 812.4 45734 ± 3621.9 44356 ± 222.2 46895 ± 1267.3	37302 ± 374.5 34979 ± 1213.1 34152 ± 1068.2 34367 ± 511.5	48460 ± 3701.1 51866 ± 4531.7 46936 ± 2358.3 46194 ± 303.3 53300 ± 27.8	51579.0 ± 215.03
6 Shikimic	112.4 ± 5.42	222.8 ± 6.57 197.3 ± 6.45 162.6 ± 4.99 206.1 ± 0.54 399.7 ± 16.65	146.9 ± 0.06 145.3 ± 0.08 205.8 ± 1.58 169.0 ± 16.20 121.3 ± 30.52	20.9 ± 2.75 54.6 ± 1.43 23.3 ± 3.06 51.6 ± 3.14 52.2 ± 1.42	18.9 ± 2.48 24.4 ± 0.54 88.8 ± 1.25 19.8 ± 0.57	16.0 ± 0.29 115.9 ± 13.0 110.0 ± 1.46 78.0 ± 3.38 47.9 ± 11.58	7.0 ± 0.29
7 Fumaric	334.0 ± 4.64	520.4 ± 23.57 579.9 ± 8.42 487.8 ± 13.24 687.6 ± 5.22 600.7 ± 0.96	928.3 ± 0.82 933.4 ± 3.90 701.7± 7.83 647.2± 25.74 889.6 ± 12.16	563.2 ± 28.36 815.0 ± 13.04 933.9 ± 12.05 1232.9 ± 4.75 1840.3 ± 16.11	568.7 ± 0.57 504.4 ± 9.23 1039.2 ± 23.72 558.9 ± 1.47	2813.1 ± 48.01 471.2 ± 23.39 670.1 ± 9.82 726.4 ± 23.28 631.7 ± 13.14	915.7 ± 0.72
Total	34669	45751 44204 48191 43138 47003	67685 72131 65156 76473 79218	66019 58133 64782 63262 61116	56210 55537 62101 60441	69036 79784 65653 68281 73096	66855

[a]Results are expressed as means of three determinations ± standard deviation.

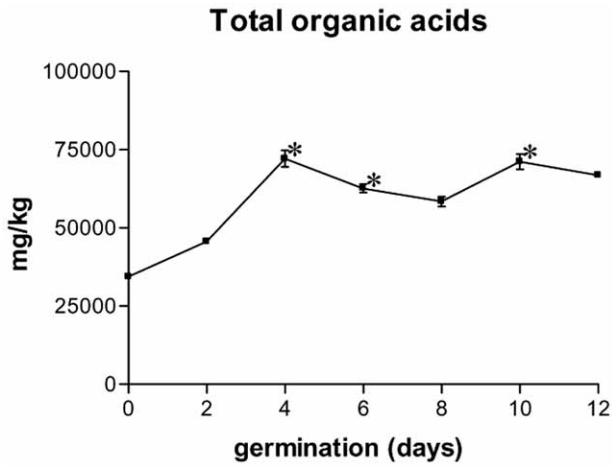

Fig. (7). Evolution in total organic acids content of *B. oleracea* sprouts with germination time. * p<0.05, compared with the previous germination time.

As observed with *B. oleracea* leaves and seeds [20, 30-31] shikimic (**6**) and fumaric (**7**) acids were present in small amounts, representing less than 0.5 and 2% of total acids, respectively (Table **3**). While fumaric acid showed behaviour similar to that exhibited by malic acid, shikimic acid displayed an opposite tendency, significantly decreasing until 6 days of germination.

4. CONCLUSIONS

The screening of the antioxidant phenolics and organic acids during 12 days sprouting of *B. oleracea* var. *costata* revealed that although the identified compounds were the same throughout the germination period, significant quantitative changes occurred, mostly during the first 4 days of germination. While organic acids greatly increased, phenolic compounds had an opposite behaviour. Besides the depletion of the seeds phenolic compounds, the synthesis of the complex kaempferol derivatives, the predominant phenolic compounds in the leaves of mature plants [30, 32], was not accomplished during this germination period. The lack of

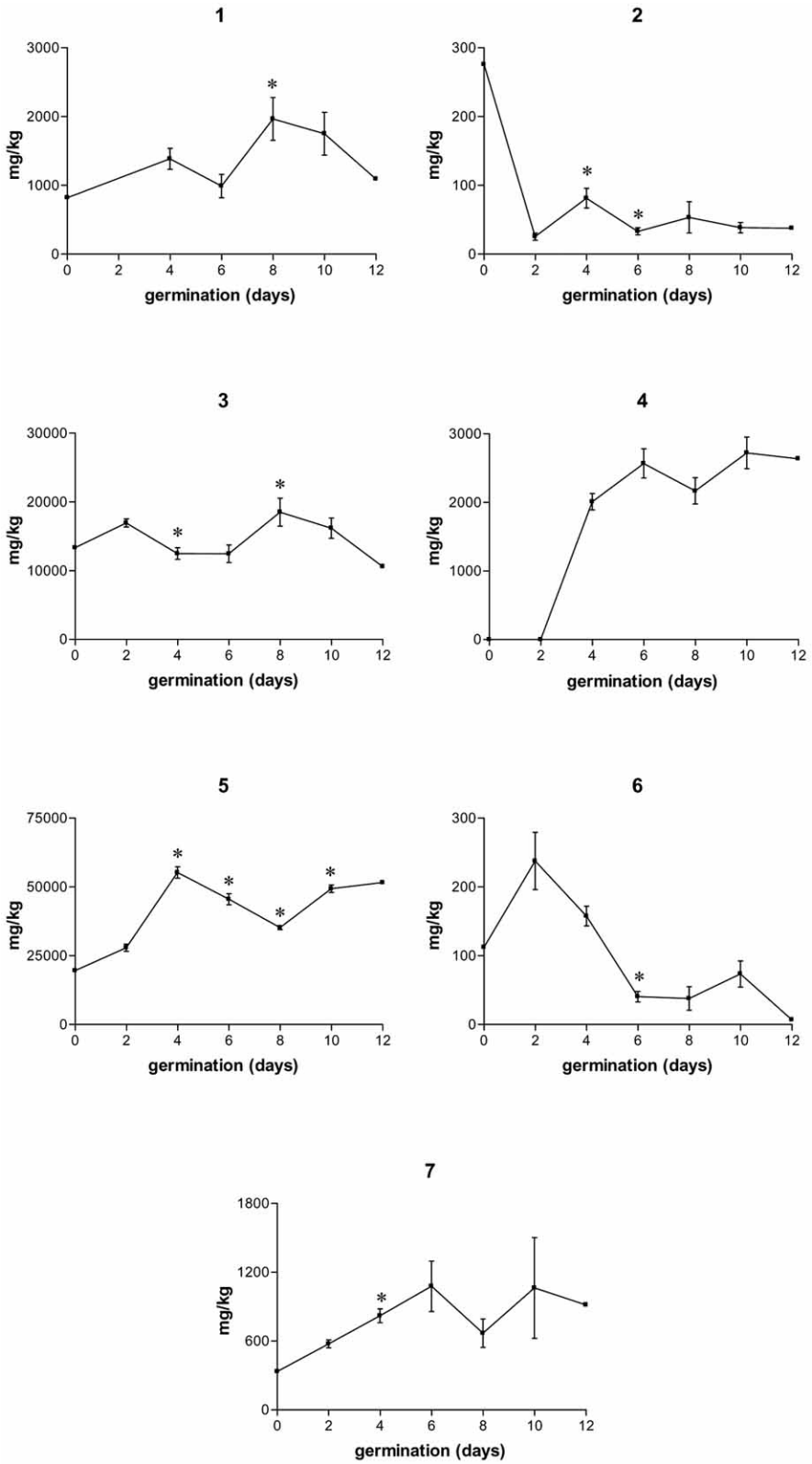

Fig. (8). Changes in individual organic acids of *B. oleracea* sprouts with germination time. Numbers of panels refer to the number of compounds (see caption of Fig. **5**). * $p<0.05$, compared with the previous germination time.

induction of flavonoid metabolism in the sprouts may be explained by the fact that the available nutrients are being required for the primary metabolism [29].

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ABBREVIATIONS

ANOVA = Analysis of variance

HPLC/DAD = High-performance liquid chromatography-diode-array detector

HPLC/UV = High-performance liquid chromatography-ultraviolet detector

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4.3. Chemical composition and antioxidant activity of tronchuda cabbage internal leaves

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Chemical composition and antioxidant activity of tronchuda cabbage internal leaves

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Abstract A phytochemical study was undertaken on the internal leaves of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC). Seventeen phenolic compounds were characterized and quantified by reversed-phase HPLC-DAD-ESI-MS^n and HPLC/DAD, respectively: quercetin 3-*O*-sophoroside-7-*O*-glucoside, 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(caffeoyl)-sophoroside-7-*O*-glucoside, sinapoyl glucoside acid, kaempferol 3-*O*-(sinapoyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside-7-*O*-glucoside, 4-*p*-coumaroylquinic acid, sinapic acid, kaempferol 3-*O*-sophoroside, 3 isomeric forms of 1,2-disinapoylgentiobiose, 1-sinapoyl-2-feruloylgentiobiose, 1,2,2-trisinapoylgentiobiose and 1,2′-disinapoyl-2-feruloylgentiobiose. Seven organic acids (aconitic, citric, ascorbic, malic, quinic, shikimic and fumaric acids) were also identified and quantified. The hot water extract of tronchuda cabbage internal leaves was investigated for its capacity to act as a scavenger of DPPH$^{\bullet}$ radical and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid), exhibiting antioxidant capacity in a concentration dependent manner against all radicals.

Keywords Tronchuda cabbage internal leaves · *Brassica oleracea* L. var. *costata* DC · Phenolics · Organic acids · Antioxidant capacity

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Inoduction

An increasing amount of evidence shows that the consumption of fruits and vegetables is, in general, beneficial to health due to the protection provided by the antioxidant compounds contained in them [1, 2]. In fact, the presence of phytochemicals, in addition to vitamins and provitamins, has been considered of great nutritional interest in the prevention of chronic diseases, such as cancer, arteriosclerosis, nephritis, diabetes mellitus, rheumatism, ischemic and cardiovascular diseases and also in the aging process, in which oxidants or free radicals are involved [3–6]. Among the natural antioxidant molecules, it can be found the liposoluble vitamins A and E, β-carotene, the water-soluble vitamin C, and a wide range of molecules generally termed phenolic compounds, including phenolic acids, flavonoids, glucosides and esters [7–9]. Synergistically or additively, these dietary antioxidants provide bioactive mechanisms to reduce free radical-induced oxidative stress [3]. Although the organism possesses such defence mechanisms as enzymes and antioxidant molecules [10, 11], oxidative stress results either from a decrease of natural cell antioxidant capacity or from an increased amount of reactive oxygen species (ROS). When the balance between oxidants and antioxidants is broken by the overproduction of free radicals beyond the organism control, it can cause irreversible oxidative damage [12].

Nowadays consumers are aware of the need for a constant supply of phytochemical-containing plants to get the most complete antioxidant support for diseases prevention. Really, through overlapping or complementary

effects, the complex mixture of the compounds present in fruits and vegetables provides a more effective protective action on health than single phytochemicals [3, 13]. This is ascribed to the fact that different plants have distinct compounds contents, with several structures, thus offering different protective mechanisms at different levels. So, to obtain optimal health benefits, it has been suggested a diet composed of a variety of phytochemical sources, such as fruits and vegetables [3, 8, 13].

Brassica vegetables, including all cabbage-like ones, are consumed in enormous quantities throughout the world and are important in human nutrition. They are reported to reduce the risks of some cancers especially due to its content of glucosinolates and their derived products [14–17], although phenolic compounds are also considered to contribute to this capacity [18–20]. Some *Brassica oleracea* varieties, namely cauliflower [21, 22], broccoli [8, 9, 22, 23] and several cabbages [2, 3, 13, 22, 24] have already been studied for their antioxidant capacity in different experimental models.

Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) plant resembles a thick-stemmed collard with large floppy leaves. Leaves are close together, round, smooth and slightly notched at the margins. Regarding the organoleptic properties, internal and external leaves are considerably different: internal leaves are pale yellow and are tender and sweeter than the dark green external leaves, which may influence the consumer's choice. Due to these characteristics internal leaves are eaten raw in salads or, most usually, cooked. As far as we know, only the phenolic composition of the external leaves has been described, consisting of complex flavonol glycosides [25], and nothing has been reported about the antioxidant capacity of tronchuda cabbage.

The objectives of this study were to define the phenolics and organic acids composition and to evaluate the antioxidant potential of tronchuda cabbage internal leaves hot-water extract, since this is the way how it is most consumed. Phenolic profile was established by reversed-phase HPLC-DAD-ESI-MS^n and HPLC-DAD analysis. Organic acids were determined by HPLC/UV. The antioxidant activity was assessed by the capacity to act as scavenger of DPPH radical and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid). A comparison with tronchuda cabbage external leaves was also undertaken.

Materials and methods

Standards and reagents

The standards were from Sigma (St. Louis, MO) and from Extrasynthése (Genay, France). Methanol, ammonium acetate and acetic acid were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA). DPPH, xanthine, xanthine oxidase (XO) grade I from buttermilk (EC 1.1.3.22), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), ferric chloride anhydrous ($FeCl_3$), ethylenediaminetetraacetic acid disodium salt (EDTA), ascorbic acid, trichloroacetic acid, thiobarbituric acid, deoxyribose, sodium hypochlorite solution with 4% available chlorine (NaOCl), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma (St. Louis, MO).

Plant material and sampling

Tronchuda cabbages were grown under organic practices, certified by the national authority (Instituto de Desenvolvimento Rural e Hidráulica), following the guidelines of Council Regulation (EEC) no 2092/91 of 24 June 1991 (organic production). Only organic fertilization was made with sheep manure and no phytosanitary treatments were applied. After harvesting in October 2004, three plants randomly selected were immediately transported to the laboratory where external and internal leaves were separated. The internal leaves were freezed and lyophilised (Modulyo 4 K Freeze Dryer Ed wards) and the three lyophilised materials were powdered, mixed and kept in an exsicator, in the dark. The same procedure was applied to the external leaves.

Sample preparation

The identification of the phenolic compounds in tronchuda cabbage internal leaves was performed using a hydromethanolic extract: the lyophilised plant material (ca. 0.5 g) was thoroughly mixed with 5 ml methanol–water (1:1), ultra-sonicated, centrifuged and filtered.

For antioxidant activity assays both internal and external leaves extracts were prepared by putting 3.0 g of lyophilised plant material in 600 ml of boiling water. The mixture was boiled for an hour and then filtered over a Büchner funnel. The resulting extracts were then lyophilised and a yield of 1.6 and 1.2 g were obtained for internal and external leaves, respectively. The lyophilised extracts were kept in an exsicator, in the dark. For organic acids analysis the lyophilised extracts were redisssolved in sulphuric acid 0.01 N. For phenolic compounds determination water was used for the redissolution of the lyophilised extracts.

HPLC analysis of organic acids

The separation was carried out as previously reported [26] with some modifications. The system consisted of an analytical HPLC unit (Gilson) with an ion exclusion column, Nucleogel Ion 300 OA (300 mm×7.7 mm) in conjunction with a column-heating device set at 30°C. Elution was carried out isocratically with sulphuric acid 0.01 N as mobile phase with a flow rate of 0.2 ml min^{-1}. The injection volume was 20 μl. Detection was performed with an UV detector set at 214 nm. Organic acids quantification was

achieved by the absorbance recorded in the chromatograms relative to external standards.

HPLC-DAD-ESI-MS^n qualitative analysis of phenolics in internal leaves

Chromatographic separations were carried out on a 250 mm×4 mm, 5-µm particle size, RP-18 LiChroCART (Merck, Darmstadt, Germany) column protected with a 4 mm×4 mm LiChroCART guard column using acetic acid 1% (A) and methanol (B) as solvents, starting with 20% B and using a gradient to obtain 50% B at 30 min and 80% B at 37 min. The flow rate was 1 ml min^{-1} and the injection volumes varied between 10 and 50 µl.

The HPLC system was equipped with an Agilent 1100 Series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, an G1313A autosampler, a G1322A degasser and a G1315B photodiode array detector controlled by a ChemStation software (Agilent, v. 08.03). Spectroscopic data from all peaks were accumulated in the range 240–400 nm, and chromatograms were recorded at 330 nm. The mass detector was a G2445A Ion-Trap Mass Spectrometer equipped with an electrospray ionisation (ESI) system and controlled by LCMSD software (Agilent, v. 4.1.). Nitrogen was used as nebulizing gas at a pressure of 65 psi and the flow was adjusted to 11 l min^{-1}. The heated capillary and voltage were maintained at 350°C and 4 kV, respectively. The full-scan mass covered the range from *m/z* 90 up to *m/z* 2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS data were acquired in the negative ionisation mode. MS^n data were achieved in the automatic mode on the more abundant fragment ion in MS^{n-1}. Tables 1–3 show the most frequent ions which characterise the fragmentation of the compounds. Other ions were found but they have not been included due to their low significance on the MS behaviour ions. The classical nomenclature for glycoconjugates was

Table 1 Rt, UV, -MS: [M-H]$^-$, -MS^2[M-H]$^-$ and -MS^3[(M-H)→Y$^7_0$ (−162)]$^-$ data of flavonoid-3-*O*-Soph-7-*O*-Glc and flavonoid-3-*O*-Soph

Compounds[a]	Rt (min)	UV (nm)	Flavonoid-3-*O*-sophoroside-7-*O*-glucoside					
			[M-H]$^-$ (*m/z*)	-MS^2[M-H]$^-$ (*m/z*) (%)	-MS^3[(M-H)→Y$^7_0$]$^-$(*m/z*) (%)			
				Y$^{7-}_0$ −162	Y$^7_0{}^{0,2}$X$^-$ (−120)	Y$^7_0$Y$^{3-}_1$ (−162)	Y$^7_0$Z$^{3-}_1$ (−180)	[Y$^7_0$Y$^3_0$−H]$^-$ (−325)[a]
1 Querc-3-Soph-7-Glc	6.5	255,265sh, 303sh,351	787.4	625 (100)		463 (4)	445 (45)	300 (100)[b]
3 Kaempf-3-Soph-7-Glc	7.8	265,303sh, 348	771.4	609 (100)	489 (45)		429 (62)	284 (100)[b]
			Flavonoid-3-*O*-Sophoroside					
			[M-H]$^-$(*m/z*)	-MS^2[M-H]$^-$ (*m/z*) (%)				
				Y$^3_1{}^-$ (−162)			Z$^3_1{}^-$ (−180)	Y$^3_0{}^-$ (−324)
11 Kaempf -3-Soph	21.3	265,300sh,349	609.3	447 (5)			429 (32)	285 (100)

[a]Querc-3-Soph-7-Glc: Quercetin-3-*O*-Sophoroside-7-*O*-Glucoside. Kaempf-3-Soph-7-Glc: Kaempferol-3-*O*-Sophoroside-7-*O*-Glucoside. sh: shoulder

[b]Fragments from homolytic cleavage of the glycosidic bond ([Y$^7_0$Y$^3_0$–H]$^{-\bullet}$) (29)

Table 2 Rt, UV, -MS: [M-H]$^-$, -MS^2[M-H]$^-$ and -MS^3[(M-H)→ (M-H-Glc)]$^-$ data of acylated derivatives from kaempferol-3-*O*-sophoroside-7-*O*-glucoside

Acylated derivatives from compound 3: Kaempferol-3-*O*-sophoroside-7-*O*-glucoside												
Compounds[a]	Rt (min)	UV (nm)	[M-H]$^-$ (*m/z*)	-MS^2[M-H]$^-$(*m/z*) (%)					-MS^3[(M-H)→ (M-H-Glc)]$^-$(*m/z*) (%)			
				−162-Glc	−308-G-*p*C	−324-G-C	−338-G-F	−368-G-S	−146-*p*.Coum	−162-Caf	−176-Fer	−206-Sinp
4 3-Caf	8.3	265, 327	933.3	771(100)		609(45)				609(100)		
6 3-Sinp	10.3	268, 333	977.4	815(100)				609(3)				609(100)
7 3-Fer	11.0	traces	947.3	785(100)			609(2)				609(93)	
8 3-*p*.Coum	11.7	traces	917.3	755(100)	609(3)				609(100)			

[a]G (Glc): Glucosyl. *p*C (*p*.Coum): *p*Coumaroyl. C (Caf): Caffeoyl. F (Fer): Feruloyl. S (Sinp): Sinapoyl

Table 3 Rt, -MS: $[M\text{-}H]^-$, $\text{-}MS^2[M\text{-}H]^-$ and $\text{-}MS^3[(M\text{-}H)\rightarrow(M\text{-}H\text{-}Acyl)]^-$ data of acylated derivatives from gentiobiosides

Compounds[a]	Rt (min)	$[M\text{-}H]^-$ (*m/z*)	$\text{-}MS^2[M\text{-}H]^-$ (*m/z*) (%)		$\text{-}MS^3[(M\text{-}H)\rightarrow(M\text{-}H\text{-}Acyl)]^-$ (*m/z*) (%)					
12 diSinp-Gentb	25.0	753.6	529(100) [M-H-224]$^-$	223 (14) [224-H]$^-$				$\text{-}MS^3[(M\text{-}H)\rightarrow(M\text{-}H\text{-}224)]^-$ 223 (100) [224-H]$^-$		
14 diSinp-Gentb	26.3	753.4	529(100) [M-H-224]$^-$	223 (5) [224-H]$^-$				223(51) [224-H]$^-$	205 (100) [206-H]$^-$	
17 diSinp-Gentb	30.0	753.4	529(100) [M-H-224]$^-$	223 (6) [224-H]$^-$				223(100) [224-H]$^-$		
13 Sinp,Fer-Gentb	26.0	723.5	529(16) [M-H-194]$^-$	499(100) [M-H-224]$^-$		$\text{-}MS^3[(M\text{-}H)\rightarrow(M\text{-}H\text{-}194)]^-$ 223 (12) [224-H]$^-$	205 (100) [206-H]$^-$	$\text{-}MS^3[(M\text{-}H)\rightarrow(M\text{-}H\text{-}224)]^-$ 193 (100) [194-H]$^-$	175 (77) [176-H]$^-$	
15 triSinp-Gentb	26.4	959.4	735 (100) [M-H-224]$^-$		529 (12) [M-H-224-206]$^-$	$\text{-}MS^3[(M\text{-}H)\rightarrow(M\text{-}H\text{-}224)]^-$ 529 (25) (−206)	511 (12) −224)	205 (100) [206-H]$^-$	$\text{-}MS^3[(M\text{-}H)\rightarrow(M\text{-}H\text{-}224\text{-}206)]^-$ 223 (100) [224-H]$^-$	205 (56) [206-H]$^-$
16 diSinp,Fer-Gentb	27.6	929.4	735 (16) [M-H-194]$^-$	705 (100) [M-H-224]$^-$		$\text{-}MS^3[(M\text{-}H)\rightarrow(M\text{-}H\text{-}194)]^-$ 511 (66) (−224)	499 (100) (−224-14)	223 (45) [224-H]$^-$	$\text{-}MS^3[(M\text{-}H)\rightarrow(M\text{-}H\text{-}224)]^-$ 511 (46) (−194)	499 (100) (−206)

[a](Gentb): Gentiobioside. (Fer): Feruloyl. (Sinp): Sinapoyl

adopted to designate the fragment ions. The ions ${}^{k,l}X_j$, $Y^n{}_j$, $Z^n{}_j$ represent fragments still containing the flavonoid aglycone, where *j* is the number of the interglycosidic bond broken, counted from the aglycon, *n* represents the position of the phenolic hydroxyl where the oligosaccharide is attached, and the *k* and *l* denote the cleavage within the carbohydrate rings. The ions obtained through the fragmentation of flavonoids with glycosylation in two different phenolic hydroxyls were labelled with a superscript number that indicates the position of these hydroxyls [27–29].

In order to enhance ionisation, mainly for the compounds **2** and **5**, ammonium acetate 10 mM with a flow of 2 ml h^{-1} was added to the eluent with a T join between the UV and the mass detector.

HPLC-DAD quantitative analysis of phenolics

Twenty microliters of the internal leaves hot-water extract were analysed using a HPLC unit (Gilson) and a RP-18 LiChroCART (Merck, Darmstadt, Germany) column (250 mm×4 mm, 5-µm particle size). The solvent system was the same as described for the qualitative analysis.

The quantification of the phenolic compounds present in external leaves hot-water extract was performed according to a described procedure [25], using a HPLC unit (Gilson) and a 250 mm×4.6 mm i.d., 5 µm Spherisorb ODS2 column (Waters, Milford). The solvent system was a mixture of formic acid 5% in water (A) and methanol (B), with a flow rate of milliliters per minute, and the gradient was as follows: 0 min: 10% B; 25 min: 20% B; 40 min: 50% B; 45 min: 50% B; 46 min: 90% B; 50 min: 90% B; 55 min: 100% B; 58 min: 100% B; and 60 min: 10% B.

In both cases detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200–400 nm, and chromatograms were recorded at 330 nm. The data were processed on Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Since standards of the compounds identified in the internal leaves hot-water extract were not commercially available quercetin 3-*O*-sophoroside-7-*O*-glucoside was quantified as rutin, 3- and 4-*p*-coumaroylquinic acids were quantified as *p*-coumaric acid, the kaempferol derivatives were quantified as kaempferol 3-*O*-rutinoside and sinapic acid derivatives as sinapic acid.

DPPH scavenging activity

The antiradical activity of the extracts was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc), by monitoring the disappearance of DPPH at 515 nm, according to a described procedure [30]. For each extract, a dilution series (five different concentrations) was prepared in a 96-well plate.

The reaction mixtures in the sample wells consisted of 25 μl hot-water extract and 200 μl DPPH radical dissolved in methanol. The plate was incubated for 30 min at room temperature. Three experiments were performed in triplicate.

Evaluation of superoxide radical scavenging activity

Antiradical activity was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc), by monitoring the effect of the lyophilised extracts on the $O_2^{\bullet-}$-induced reduction of NBT at 562 nm. The direct reduction of NBT by the extracts was checked without the superoxide radical generation systems and no effect was observed.

Non-enzymatic assay

Superoxide radicals were generated by the NADH/PMS system according to a described procedure [31]. All components were dissolved in phosphate buffer 19 mM, pH 7.4. Three experiments were performed in triplicate.

Enzymatic assay

Superoxide radicals were generated by the xanthine/xanthine oxidase (X/XO) system following a described procedure [31]. Xanthine was dissolved in NaOH 1 μM and subsequently in phosphate buffer 50 mM with EDTA 0.1 mM, pH 7.8, xanthine oxidase in EDTA 0.1 mM and the other components in phosphate buffer 50 mM with EDTA 0.1 mM, pH 7.8. Three experiments were performed in triplicate.

Effect on xanthine oxidase activity

The effect of the lyophilised extracts on xanthine oxidase activity was evaluated by measuring the formation of uric acid from xanthine in a double beam spectrophotometer (Heλios α, Unicam), at room temperature. The reaction mixtures contained the same proportion of components as in the enzymatic assay for superoxide radical scavenging activity, except NBT, in a final volume of 750 μl. The absorbance was measured at 295 nm for 2 min. Three experiments were performed in triplicate.

Hydroxyl radical assay

The deoxyribose method for determining the scavenging effect of the hot-water extracts on hydroxyl radicals was performed according to a described procedure [32] in a double beam spectrophotometer (Heλios α, Unicam). Reaction mixtures contained 50 μM ascorbic acid, 40 μM $FeCl_3$, 2 mM EDTA, 2.8 mM H_2O_2, 2.8 mM deoxyribose and lyophilised extracts. All components were dissolved in KH_2PO_4-KOH buffer 10 mM, pH 7.4. This assay was also performed either without ascorbic acid or EDTA, in order to evaluate the extracts pro-oxidant and metal chelation potential, respectively. Three experiments were performed in triplicate.

Hypochlorous acid scavenging activity

The inhibition of hypochlorous acid-induced 5-thio-2-nitrobenzoic acid (TNB) oxidation to 5,5'-dithiobis(2-nitrobenzoic acid) was performed according to a described procedure [32], in a double beam spectrophotometer (Heλios α, Unicam). Hypochlorous acid and TNB were prepared immediately before use. Scavenging of hypochlorous acid was ascertained by using lipoic acid as a reference scavenger, which scavenged HOCl in a concentration dependent manner (data not shown). Three experiments were performed in triplicate.

Results and discussion

Characterization of the internal leaves phenolic compounds

The screening by HPLC-DAD-ESI-MS^n of the hydromethanolic extract of tronchuda cabbage internal leaves (Fig. 1A) revealed the presence of several hydroxycinnamic acid derivatives, with the exception of compounds **1**, **3** and **11**, which UV and MS spectra (Table 1) coincided with those of quercetin 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside-7-*O*-glucoside and kaempferol 3-*O*-sophoroside, respectively. These compounds had already been characterized in other *Brassica* vegetables, namely cauliflower [33] and broccoli [34], and their MS fragmentation allows them to be differentiated from other possible isomers [35]. Compounds **4**, **6**, **7** and **8** are monoacylated derivatives of compound **3**, being caffeic, sinapic, ferulic and *p*-coumaric acids linked to the sophorose, respectively (Table 2). These compounds had also been reported in the previously mentioned *Brassica* species and exhibit a characteristic MS fragmentation pathway [34].

The HPLC-DAD-ESI-MS^n analysis showed an abundance of non-flavonoidic hydroxycinnamic acid derivatives. Thus, in the first part of the chromatogram (Fig. 1A) compound **2** is the main one, which fragmentation (-MS: 337 $[M-H]^-$, -MS^2 $[M-H]^-$: 163(100%)) is coincident with that of 3-*p*-coumaroylquinic acid [36]. Sinapoyl glucoside acid (**5**) (-MS: 385 $[M-H]^-$, -MS^2: 247 (51%), 223 ($[M-H-glucosyl]^-$, 100%), 205 ($[sinapoyl-H]^-$, 70%)) was also detected.

In the second part of the chromatogram (Fig. 1A) some compounds corresponding to sinapic and ferulic acids esterified with a dihexose were noticed (compounds **12**–**17**). Several conjugates of gentiobiose (glucosil 1→6 glucose)

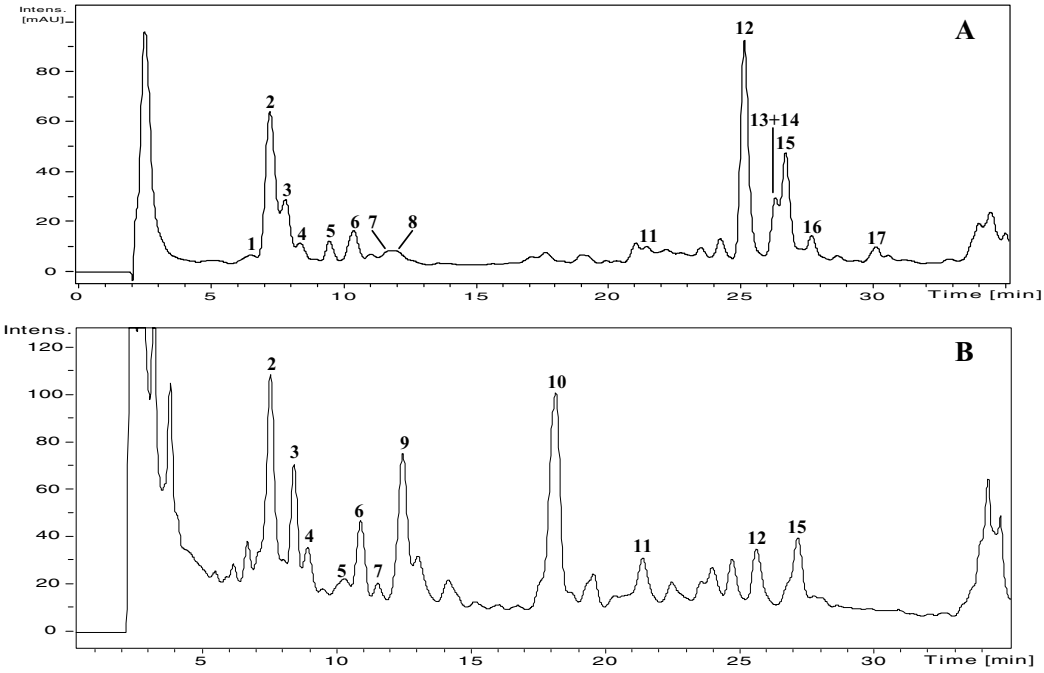

Fig. 1 HPLC-DAD phenolic profile of tronchuda cabbage internal leaves (**A**) hydro-methanolic extract and (**B**) hot-water extract. Detection at 330 nm. Peaks: (**1**) quercetin 3-*O*-sophoroside-7-*O*-glucoside; (**2**) 3-*p*-coumaroylquinic acid; (**3**) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (**4**) kaempferol 3-*O*-(caffeoyl)-sophoroside-7-*O*-glucoside; (**5**) sinapoyl glucoside acid; (**6**) kaempferol 3-*O*-(sinapoyl)-sophoroside-7-*O*-glucoside; (**7**) kaempferol 3-*O*-(feruloyl)-sophoroside-7-*O*-glucoside; (**8**) kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside-7-*O*-glucoside; (**9**) 4-*p*-coumaroylquinic acid; (**10**) sinapic acid; (**11**) kaempferol 3-*O*-sophoroside; (**12**) 1,2-disinapoylgentiobiose; (**13**) 1-sinapoyl-2-feruloylgentiobiose; (**14**) isomer of 1,2-disinapoylgentiobiose; (**15**) 1,2,2′-trisinapoylgentiobiose; (**16**) 1,2′-disinapoyl-2-feruloyl-gentiobiose; (**17**) isomer of 1,2-disinapoylgentiobiose

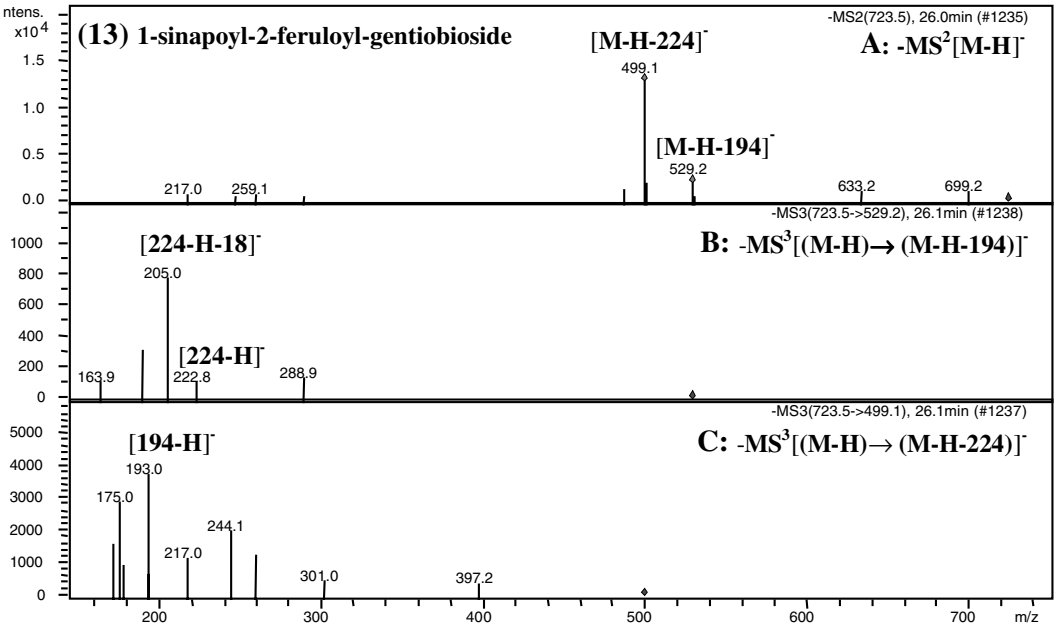

Fig. 2 Negative MS^n analysis of 1-sinapoyl-2-feruloylgentiobiose (**13**). (**A**) $MS^2[M\text{-}H]^-$; (**B**) $MS^3[(M\text{-}H)\rightarrow (M\text{-}H\text{-}Fer)]^-$; (**C**) $MS^3[(M\text{-}H)\rightarrow (M\text{-}H\text{-}Fer\text{-}Sinp)]^-$

and hydroxycinnamic acids had been characterized before in broccoli [37] and, therefore, the compounds detected in this work may be coincident with them. In addition, this type of compounds is very common in *Brassica* species [33, 34, 38]. From the study of the distinct MS^n fragmentation pathways (Table 3) it could be observed, in all cases, the loss of 224 amu from the deprotonated molecular ion, corresponding to sinapic acid. Those compounds that besides sinapic acid presented ferulic acid (compounds **13** and **16**) also displayed the loss of this acid (194 amu)

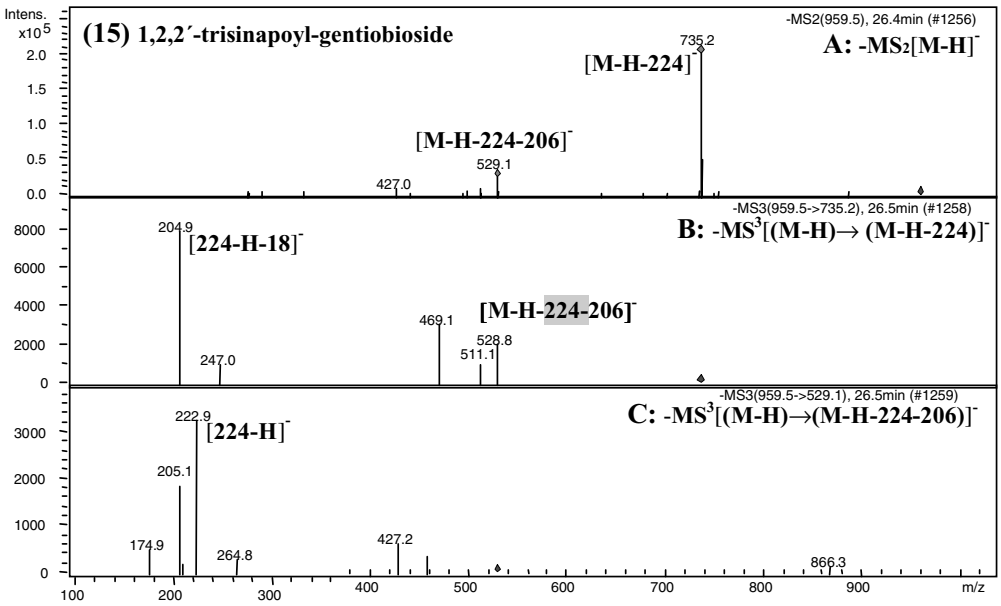

Fig. 3 Negative MS^n analysis of 1,2,2′-trisinapoylgentiobiose (**15**). (**A**) $MS^2[M-H]^-$; (**B**) $MS^3[(M-H\rightarrow(M-H-sinp]^-$; (**C**) $MS^3[(M-H)\rightarrow(M-H-Sinp-Sinp)]^-$

(Fig. 2A, Table 3). In $MS^3[(M-H)\rightarrow$ (M-H-Acyl)]$^-$ (Table 3) it could be observed, besides an additional loss of acyls, the ions corresponding to the acids: *m*/*z* 223 [sinapic acid-H]$^-$, 205 [sinapic acid-H-18]$^-$, 193 [ferulic acid-H]$^-$, 175 [ferulic acid-H-18]$^-$ (Figs. 2B, C, 3B and C). Other ions resulting from the partial fragmentation of the sugars were observed but not included in Table 3. Tentatively, we can consider that the detected compounds are coincident with those previously described [37] and were identified as 1,2-disinapoylgentiobiose (**12**), 1-sinapoyl-2-feruloylgentiobiose (**13**), 1,2,2′-trisinapoylgentiobiose (**15**), 1,2′-disinapoyl-2-feruloylgentiobiose (**16**). Compounds **14** and **17** are 1,2-disinapoylgentiobiose isomers. In the hydroalchoolic extract synapic acid (**10**) was also detected in trace amounts (Fig. 1A).

The aqueous lyophilised extract of tronchuda cabbage internal leaves presented a similar composition. However, in this extract it were not found quercetin 3-*O*-sophoroside-7-*O*-glucoside (**1**), kaempferol 3-*O*-(p-coumaroyl)-sophoroside-7-*O*-glucoside (**8**), 1-sinapoyl-2-feruloylgentiobiose (**13**) and the isomers of 1,2-disinapoylgentiobiose (**14** and **17**). Besides, in this aquous extract 4-*p*-coumaroylquinic acid (**9**) was present (Fig. 1B).

All these compounds are identified in tronchuda cabbage for the first time, with the exceptions of kaempferol

Table 4 Phenolic composition of tronchuda cabbage internal leaves hot-water extract (mg of phenolic compound kg^{-1} of lyophilised extract)[a]

	Phenolic compound	Mean	SD
1	Quercetin 3-*O*-sophoroside-7-*O*-glucoside	nd	
2	3-*p*-Coumaroylquinic acid	189.0	1.4
3	Kaempferol 3-*O*-sophoroside-7-*O*-glucoside	287.8	8.8
4	Kaempferol 3-(cafeoyl)-sophoroside-7-*O*-glucoside	120.5	3.7
5	Sinapoyl glucoside acid	25.7	0.2
6	Kaempferol 3-(sinapoyl)-sophoroside-7-*O*-glucoside	180.7	5.5
7	Kaempferol 3-(feruloyl)-sophoroside-7-*O*-glucoside	53.6	1.6
8	Kaempferol 3-(p-coumaroyl)-sophoroside-7-*O*-glucoside	nd	
9	4-*p*-Coumaroylquinic acid	126.0	0.9
10	Sinapic acid	180.1	1.1
11	Kaempferol 3-*O*-sophoroside	100.4	3.1
12	1,2-Disinapoylgentiobioside	51.5	0.3
13	1-Sinapoyl-2-feruloylgentiobiose	nd	
14	Isomer of 1,2-disinapoylgentiobioside	nd	
15	1,2,2′-trisinapoylgentiobiose	62.9	0.4
16	1-2′-disinapoyl-2-feruloylgentiobiose	11.4	0.1
17	Isomer of 1,2-disinapoylgentiobioside	nd	
		Σ1389.6	

[a]Results are expressed as mean of three determinations. SD standard deviation, ∑, sum of the determined phenolic compounds

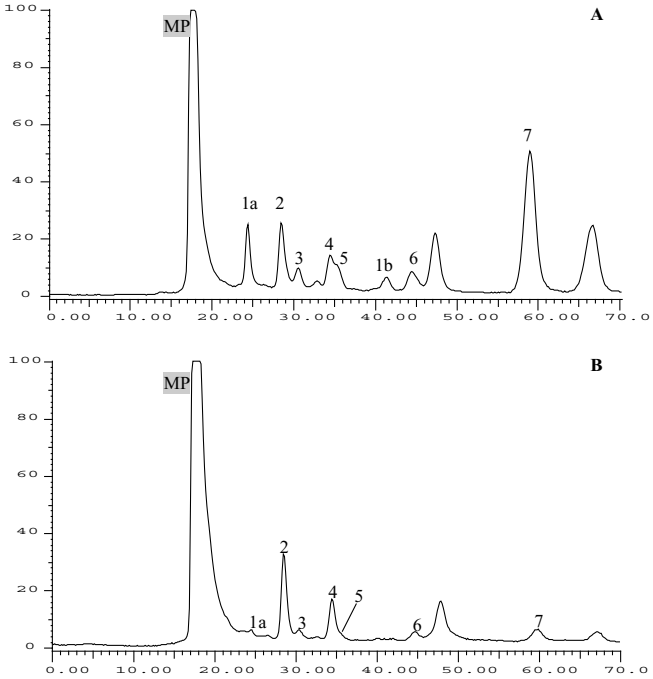

Fig. 4 HPLC-UV organic acid profile of tronchuda cabbage (**A**) internal and (**B**) external leaves hot-water extracts. Detection at 214 nm. Peaks: (MP) mobile phase; (**1a** and **1b**) aconitic acid isomers; (**2**) citric acid; (**3**) ascorbic acid; (**4**) malic acid; (**5**) quinic acid; (**6**) shikimic acid; (**7**) fumaric acid

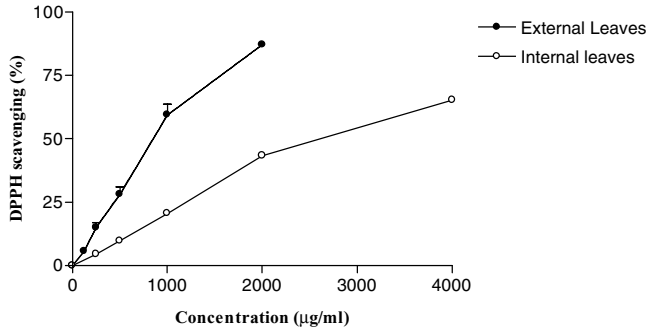

Fig. 5 Effect of tronchuda cabbage internal and external leaves on DPPH reduction. Values show mean ± SE from three experiments performed in triplicate

3-*O*-sophoroside-7-*O*-glucoside (**3**) and kaempferol 3-*O*-sophoroside (**11**), previously described in its external leaves [25].

Internal leaves phenolic compounds quantitative analysis

In order to a get a better characterization of the aqueous lyophilised extract of tronchuda cabbage internal leaves used in antioxidant assays, its phenolic compounds were quantified by HPLC/DAD. The results showed a high content of phenolics (ca. 1.4 g kg^{-1}, dry basis) (Table 4), being kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**3**) the main compound (ca. 21% of total identified compounds),

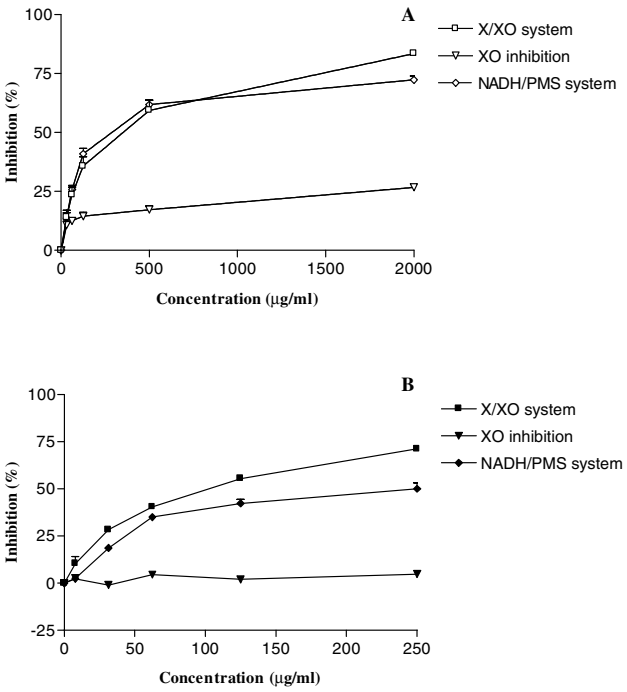

Fig. 6 Effect of tronchuda cabbage (**A**) internal and (**B**) external leaves against superoxide radical generated in an enzymatic and non-enzymatic systems and on XO activity. Values show mean ± SE from three experiments performed in triplicate

followed by 3-*p*-coumaroylquinic acid (**2**) (ca. 14% of total phenolics). 1,2′-Disinapoyl-2-feruloylgentiobiose (**16**) was present in the lowest amount (ca. 1% of total identified compounds).

Sinapic acid (**10**), which was detected only in vestigial amounts in the hydro-methanolic extract, represented 13% of the identified phenolics in the aqueous lyophilised extract. The presence of higher amounts of sinapic acid and the existence of 4-*p*-coumaroyl quinic acid (**9**) in the hot-water extract may be attributed to a higher solubility of these compounds in the boiling water. Decomposition of the sinapic acid derivatives can partially explain the differences observed in the chromatograms of both methanolic and hot-water extracts.

Identification and quantification of organic acids by HPLC/UV

Tronchuda cabbage internal leaves presented a chemical profile composed by seven identified organic acids: aconitic, citric, ascorbic, malic, quinic, shikimic and fumaric acids (Fig. 4A). All these compounds are described for the first time in this cabbage. None of the samples presented oxalic, ketoglutaric, succinic, lactic, acetic, pyruvic, malonic or tartaric acids. The lyophilised extract exhibited a high content of organic acids (ca. 23 g kg^{-1}), in which citric acid was the main compound, representing ca. 43% of total identified organic acids, followed by the pair malic plus quinic acids (ca. 28% of total acids)

Table 5 Organic acids in tronchuda cabbage leaves hot-water extracts (mg of organic acid kg^{-1} of lyophilised extract)[a]

	Organic acid	Internal leaves		External leaves	
		Mean	SD	Mean	SD
1	Aconitic	191.1	3.7	21.7	8.2
2	Citric	9974.6	68.2	8130.7	421.2
3	Ascorbic	6020.1	143.4	8754.1	517.4
4+5	Malic + Quinic	6626.4	164.8	8604.7	974.6
6	Shikimic	35.0	1.0	19.7	0.5
7	Fumaric	407.6	1.8	14.2	0.1
	Σ	23254.8		25545.1	

[a]Results are expressed as means of three determinations; SD standard deviation, ∑, sum of the determined organic acids

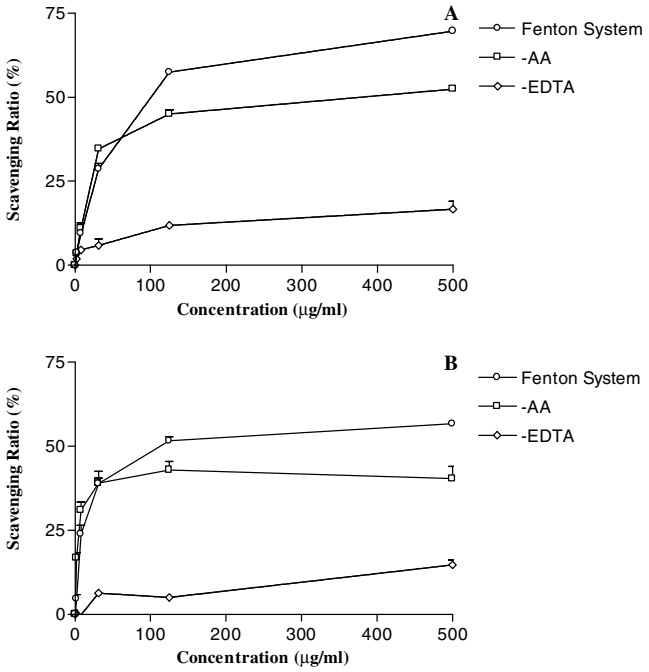

Fig. 7 Tronchuda cabbage (**A**) internal and (**B**) external leaves non-specific hydroxyl radical scavenging activity, pro-oxidant activity (-AA) and specific hydroxyl radical scavenging (-EDTA). Values show mean ± SE from three experiments performed in triplicate

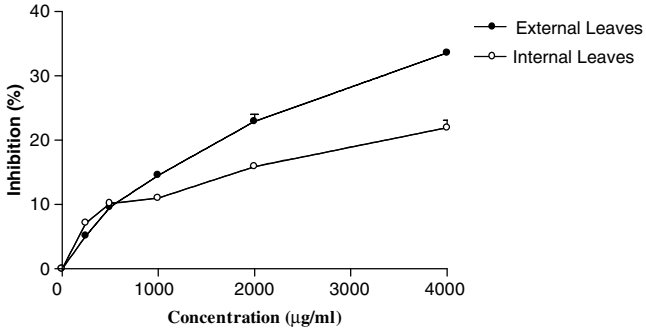

Fig. 8 Effect of tronchuda cabbage internal and external leaves on the oxidation of TNB by HOCl. Values show mean ± SE from three experiments performed in triplicate

(Table 5). Shikimic acid was the compound present in minor amounts, accounting for ca. 0.2% of compounds (Table 5).

Antioxidant activity

The DPPH• assay constitutes a screening method currently used to provide basic information on the antiradical activity of extracts. Reduction of DPPH• by antioxidants leads to a loss of absorbance at 515 nm [39]. In the present work the lyophilised extract of tronchuda cabbage internal leaves

Table 6 Phenolic composition of tronchuda cabbage external leaves hot-water extract (mg of phenolic compound kg^{-1} of lyophilised extract)[a]

	Phenolic compound	Mean	SD
2	3-*p*-Coumaroylquinic acid	481.8	8.6
3	Kaempferol 3-*O*-sophoroside-7-*O*-glucoside	852.2	14.0
11	Kaempferol 3-*O*-sophoroside	797.4	31.4
18 + 19	Kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside + kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside	452.6	12.3
20	Kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside	445.3	6.1
21	Kaempferol 3-*O*-sophoroside-7-*O*-sophoroside	2788.4	48.9
22	Kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside	1682.0	21.2
23	Kaempferol 3-*O*- (feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside	1886.0	45.1
24	Kaempferol 3-*O*-sophorotrioside	718.9	7.2
25	Kaempferol 3-*O*-(sinapoyl)-sophoroside	1244.0	62.8
26+27	Kaempferol 3-*O*-(feruloyl)-sophorotrioside + kaempferol 3-O-(feruloyl)-sophoroside	1988.8	33.1
28	Kaempferol 3-*O*-glucoside	nq	–
		Σ13337.4	

[a]Results are expressed as mean of three determinations. SD standard deviation, ∑, sum of the determined phenolic compounds

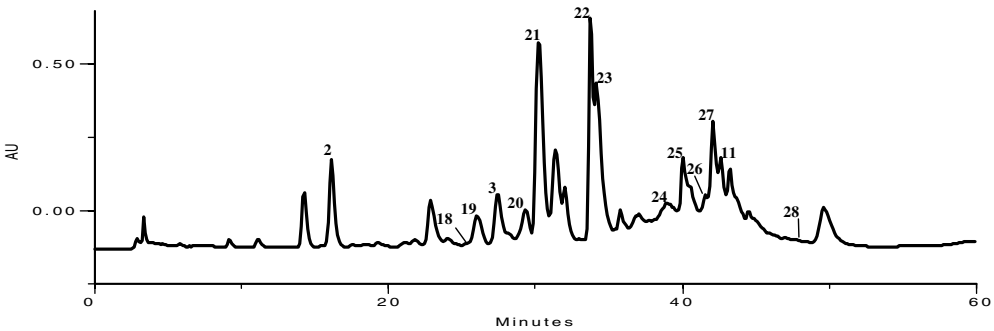

Fig. 9 HPLC-DAD phenolic profile of tronchuda cabbage external leaves hot-water extract. Detection at 330 nm. Peaks: (**2**) 3-*p*-coumaroylquinic acid; (**3**) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (**11**) kaempferol 3-*O*-sophoroside; (**18**) kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside; (**19**) kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside; (**20**) kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside; (**21**) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside; (**22**) kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside; (**23**) kaempferol 3-*O*- (feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside; (**24**) kaempferol 3-*O*-sophorotrioside; (**25**) kaempferol 3-*O*-(sinapoyl)-sophoroside; (**26**) kaempferol 3-*O*-(feruloyl)-sophorotrioside; (**27**); kaempferol 3-*O*-(feruloyl)-sophoroside; (**28**) kaempferol 3-*O*-glucoside

displayed a concentration-dependent antioxidant potential, measured by the DPPH assay (IC_{25} at 1192 μg ml^{-1}), although with less effectiveness than external leaves (IC_{25} at 440 μg ml^{-1}) (Fig. 5).

Tronchuda cabbage internal leaves scavenged X/XO-generated superoxide radical in a concentration dependent manner (Fig. 6A), with an IC_{50} at 351 μg ml^{-1}, but its external leaves exhibited stronger antiradical capacity (IC_{50} at 102 μg ml^{-1}) (Fig. 6B). Since an inhibitory effect on the enzyme itself would also lead to a decrease in NBT reduction [31], the effect of the lyophilised extracts on the metabolic conversion of xanthine to uric acid was checked. The results demonstrate that the internal leaves extract had a weak inhibitory effect on XO, which was concentration dependent (IC_{10} at 273 μg ml^{-1}) (Fig. 6A), while external leaves had no effect on the enzyme (Fig. 6B). The capacity of both lyophilized extracts to scavenge superoxide radicals in a concentration-dependent way was confirmed when this radical was generated by a chemical system composed by PMS, NADH and oxygen. The results indicated that internal leaves, with an IC_{25} at 101 μg ml^{-1} (Fig. 6A), had lower ability to scavenge superoxide radical than external leaves (IC_{25} at 43 μg ml^{-1}) (Fig. 6B). So, the antioxidant activity exhibited by the internal leaves in the enzymatic assay is achieved by their capacity to act as both superoxide radical scavengers and XO inhibitors.

Tronchuda cabbage internal leaves extract also exhibited a potent scavenging activity for hydroxyl radical in a concentration dependent manner (Fig. 7A), with an IC_{25} at 27 μg/ml, although less efficient than external leaves extract (IC_{25} at 10 μg/ml) (Fig. 7B). If we omit ascorbate in the reaction mixture, and if prooxidant compounds are present, they will be able to redox cycle the metal ion required for hydroxyl generation, and thus increase the radical production [40]. In order to evaluate the pro-oxidant potential of the extracts, we omitted ascorbic acid, and we found that both lyophilised extracts were not effective substitutes for ascorbic acid (Fig. 7). Thus, tronchuda cabbage internal and external leaves do not act as pro-oxidants. Some compounds inhibit deoxyribose degradation in this assay, not by reacting with hydroxyl radicals, but because they present ion-binding capacity and can withdraw the iron ions and render them inactive or poorly active in Fenton reactions [41]. Attending to this fact, the assay was also performed in the absence of EDTA in order to check the ability of the extracts to chelate iron ions. In the assay performed under these conditions both internal and external leaves lyophilised extracts had a similar behaviour, exhibiting a weak ability to chelate iron ions (Fig. 7).

The oxidizing properties of HOCl induce the conversion of TNB (λ_{max}=412 nm) to DTNB (λ_{max}=315 nm). In the present assay a HOCl scavenger inhibits the oxidation of TNB into DTNB [42]. Tronchuda cabbage internal leaves lyophilized extract exhibited a weak HOCl scavenging activity, in a concentration-dependent manner, as shown in Fig. 8. The external leaves revealed to have higher antioxidant capacity against HOCl (Fig. 8).

Despite the antioxidant capacity exhibited by tronchuda cabbage internal leaves, in general terms, and according to the results obtained in all assays, they exhibited lower antioxidant potential than external leaves. This can be ascribed to the higher content of both phenolics and organic acids in the external leaves (Tables 5 and 6), which are known to have antioxidant activity [43]. In addition, the qualitative phenolic composition is also different: in the external leaves extract, with the exceptions of 3-*p*-coumaroylquinic acid (**2**), kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**3**) and kaempferol 3-*O*-sophoroside (**11**), several phenolic compounds were identified, namely flavonol glycosides, distinct from those detected in the internal leaves (Fig. 9). Either flavonol glycosides [44, 45] or hydroxycinnamic esters [46] have demonstrated antioxidant activity. However, as the content of flavonol glycosides is higher in external leaves than in internal ones (95 and 54% of total phenolic compounds, respectively), this class of phenolics may contribute the most for the effects observed. On the other hand, the higher amount of acylated flavonols, namely caffeoyl derivatives, in the external leaves (Table 6) might also explain their potent antioxidant capacity. These compounds are reported

to have high scavenging ability due to the presence of an *o*-dihydroxy structure in the caffeoyl moiety, which confers great stability to the radical form and participates in the electron delocalisation [44].

Concerning the organic acids, despite the presence of the previously mentioned seven compounds (Fig. 4), quantitative differences were noticed between internal and external leaves (Table 5), which may influence the antioxidant potential. Citric acid was the main compound in the external leaves, corresponding to 43% of total identified acids, followed by the pair malic plus quinic acids (28%), while internal leaves presented ascorbic acid and the pair malic plus quinic acids in the highest amount (34%, each). Thus, it seems that citric acid may have an important role in the antioxidant potential of tronchuda cabbage, as can be seen by the higher capacity exhibited by the external leaves. In fact, citric acid is known to protect ascorbic acid from metal-catalysed oxidation and to function as a synergist with other antioxidants [43].

In conclusion, the results obtained in the present work denote that tronchuda cabbage internal leaves may constitute a good source of health promoting compounds, namely flavonoids and organic acids. In addition, and as far as we know, this is the first report considering the antioxidant potential of the species, suggesting that it could be useful in the prevention of diseases in which free radicals are implicated.

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4.4. Influence of two fertilization regimens on the amounts of organic acids and phenolic compounds of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC)

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Influence of Two Fertilization Regimens on the Amounts of Organic Acids and Phenolic Compounds of Tronchuda Cabbage (*Brassica oleracea* L. Var. *costata* DC)

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A phytochemical study was undertaken on tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) cultivated under conventional and organic practices and collected at different times. Six organic acids (aconitic, citric, ascorbic, malic, shikimic, and fumaric acids) were identified and quantified by HPLC-UV. Qualitative and quantitative differences were noted between internal and external leaves. Analysis of the phenolics of the internal leaves was achieved by HPLC-DAD, and the phenolic profile obtained was revealed to be distinct from that of the external leaves. By this means were identified and quantified 11 compounds: 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside, two isomeric forms of 1,2-disinapoylgentiobiose, 1-sinapoyl-2-feruloylgentiobiose, 1,2,2′-trisinapoylgentiobiose, and 1,2′-disinapoyl-2-feruloylgentiobiose. In general, internal leaves exhibited more constant chemical profiles.

KEYWORDS: Tronchuda cabbage; *Brassica oleracea* L. var. *costata* DC; organic acids; phenolic compounds; organic and conventional production

INTRODUCTION

Nowadays consumers are aware of the need for a constant supply of phytochemical-containing plants to get optimal health benefits. Vegetables form an essential part of a well-balanced diet. *Brassica* vegetables, including all cabbage-like ones, are very popular, being consumed in enormous quantities all over the world. *Brassica* species are reported to possess cancer preventive activity, due to glucosinolates and their derived properties (*1*−*3*). Their leaves have little starch, sugar, or fat, their fiber is useful; they contain some vitamin E and a range of B vitamins, and they present significant carotenes and ascorbic acid contents. Within *Brassica* genus, the *B. oleracea* species has evolved into a number of varieties of which different parts of the plant have become the edible constituents (*1*). Although essentially temperate, *B. oleracea* forms are grown in other regions throughout the world (*1*). Tronchuda cabbage (*B. oleracea* L. var. *costata* DC) is known to be a cultivar well adapted to the soil and climate conditions and generally grown with little or no agrochemical input (*4*). It is very important in the Portuguese diet and agricultural systems. Tronchuda cabbage exhibits large floppy leaves, which are close together, round, smooth, and slightly notched at the margins. Its dark green external leaves are rather bitter and tough and are usually prepared by boiling. The internal leaves are pale yellow, tender, and sweeter than the external ones, being consumed raw or, most usually, cooked.

Organic acids and phenolic compounds are known to contribute to the organoleptic characteristics of fruits and vegetables (*5*). These compounds have been used for the quality control of several matrices (*6*−*10*). In addition, they may also exert a protective role against various diseases due to their antioxidant potential (*11*). The phenolic composition of tronchuda cabbage external leaves has already been characterized (*12*). The phenolic compounds of the internal leaves were identified by HPLC-DAD-MS/MS-ESI (*13*), but no study concerning their variation has been done. The organic acids present in the external and internal leaves have been characterized (*13*), but their variation was not analyzed.

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Table 1. Quantification of Tronchuda Cabbage Organic Acids (Milligrams per Kilogram, Dry Basis)[a]

sample	collection date	leaves	cultivation[b]	compound: aconitic acid (t_R 24.2 min)	citric acid (t_R 28.7 min)	ascorbic acid (t_R 29.8 min)	malic acid (t_R 34.3 min)	shikimic acid (t_R 46.5 min)	fumaric acid (t_R 57.7 min)	total
1	Oct 2002	external	O		7429.8 (1019.6)	9778.3 (358.0)	9133.5 (1087.1)	143.5 (7.5)	21.9 (0.7)	26507.0
2	Oct 2002	external	C	61.0 (0.9)	18980.4 (1112.2)	28843.7 (1344.4)	25904.3 (307.6)	171.3 (12.7)	34.6 (0.6)	73995.2
3	Nov 2002	external	O		9749.6 (576.2)	10978.7 (546.9)	11183.7 (164.8)	124.9 (5.7)	8.6 (1.2)	32045.6
4	Nov 2002	external	C	78.0 (22.4)	9362.6 (458.9)	28441.8 (2914.0)	22184.6 (1073.3)	226.1 (15.3)	27.8 (0.3)	60321.0
5	Dec 2002	external	O	64.8 (6.1)	5041.3 (946.8)	31179.1 (4054.5)	39459.0 (2357.6)	118.3 (16.9)	1.3 (0.1)	75863.8
6	Dec 2002	external	C	76.3 (16.4)	9962.8 (2164.2)	30634.3 (3468.0)	28385.9 (1391.4)	172.7 (2.2)	2.5 (0.2)	69234.5
7	Jan 2003	external	O		3017.6 (182.5)	15516.5 (3519.2)	12030.4 (583.1)	138.0 (12.5)	25.8 (1.2)	30728.3
8	Jan 2003	external	C	93.0 (16.1)	8282.4 (392.8)	30906.2 (7289.2)	32207.5 (2452.6)	232.1 (2.9)	7.9 (3.8)	71729.0
9	Oct 2002	internal	O	114.2 (10.1)	9681.7 (12.8)	31069.4 (632.4)	33449.7 (149.9)	269.2 (2.3)	41.6 (1.1)	74625.9
10	Oct 2002	internal	C	20.2 (1.1)	4689.9 (29.8)	nq[c]	32270.5 (193.6)	257.4 (3.4)	26.1 (16.3)	37264.1
11	Nov 2002	internal	O	176.4 (13.7)	6912.3 (21.0)	15852.7 (930.4)	17620.8 (688.8)	155.8 (1.6)	143.3 (2.8)	40861.4
12	Nov 2002	internal	C	59.7 (0.7)	5198.1 (305.0)	nq	32710.7 (924.3)	216.5 (0.8)	26.2 (1.9)	38211.2
13	Dec 2002	internal	O	135.4 (2.7)	8525.4 (328.0)	25043.9 (181.6)	52869.6 (582.8)	200.9 (3.9)	53.9 (3.2)	86829.1
14	Dec 2002	internal	C	83.7 (8.1)	4108.7 (76.7)	nq	6735.9 (657.0)	178.7 (4.6)	58.8 (1.0)	11165.8
15	Jan 2003	internal	O	113.8 (18.2)	5560.4 (2.4)	36225.6 (3005.6)	10080.3 (1069.9)	248.9 (16.1)	91.3 (5.2)	52320.2
16	Jan 2003	Internal	C	101.8 (42.0)	9448.6 (1701.5)	21030.6 (5206.2)	5907.2 (625.5)	303.6 (3.7)	62.6 (0.1)	36854.4

[a] Results are expressed as mean (standard deviation) of three determinations. [b] O, organic; C, conventional. [c] Not quantified.

Table 2. Quantification of Tronchuda Cabbage Internal Leaves Phenolic Compounds (Milligrams per Kilogram, Dry Basis)[a]

sample	collection date	leaves	cultivation[c]	compound[b]: **1** (t_R 14.1 min)	**2** (t_R 26.3 min)	**3** (t_R 27.3 min)	**4** (t_R 31.8 min)	**5** (t_R 32.6 min)	**6** (t_R 40.1 min)	**7** + **8** + **9** + **10** (t_R 43.6–45.0 min)	**11** (t_R 46.0 min)	total
9	Oct 2002	internal	O	69.7 (3.8)		7.4 (0.2)	3.5 (0.7)	0.9 (0.8)	2.9 (0.1)	85.0 (2.5)	3.1 (0.1)	172.5
10	Oct 2002	internal	C	61.0 (0.1)		14.1 (0.6)	1.5 (0.0)	14.5 (0.2)	1.4 (0.0)	54.1 (4.3)		146.6
11	Nov 2002	internal	O	23.2 (1.0)		2.7 (0.4)	nq[d]	nq		42.1 (0.3)	2.5 (0.2)	70.5
12	Nov 2002	internal	C	57.4 (8.7)		6.3 (1.1)			0.9 (0.0)	33.1 (6.1)	1.4 (0.2)	99.1
13	Dec 2002	internal	O	34.3 (0.6)		3.7 (0.1)			1.0 (0.0)	32.0 (0.1)	0.0 (0.0)	71.2
14	Dec 2002	internal	C	17.7 (2.2)		1.5 (0.0)			2.7 (0.1)	14.6 (2.7)	0.3 (0.1)	36.8
15	Jan 2003	internal	O	33.3 (2.2)	nq	5.2 (0.1)	2.0 (0.1)	3.8 (0.1)	4.0 (0.7)	73.3 (0.2)	0.7 (0.9)	122.3
16	Jan 2003	internal	C	16.7 (2.8)	12.2 (0.6)		28.0 (5.4)	17.9 (1.3)	13.8 (0.9)	9.0 (0.9)		97.6

[a] Results are expressed as mean (standard deviation) of three determinations. [b] Identified according to ref *13*: **1**, 3-*p*-coumaroylquinic acid; **2**, kaempferol 3-*O*-sophoroside-7-*O*-glucoside; **3**, kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside; **4**, kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside; **5**, kaempferol 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside; **6**, kaempferol 3-*O*-sophoroside; 7, 1,2-disinapoylgentiobiose; **8**, 1-sinapoyl-2-feruloylgentiobiose; **9**, isomer of 1,2-disinapoylgentiobiose; **10**, 1,2,2′-trisinapoylgentiobiose; **11**, 1,2′-disinapoyl-2-feruloylgentiobiose. [c] O, organic; C, conventional. [d] Not quantified.

The combination of several environmental factors may lead either to irreversible injuries or to the induction of reactions resulting in the plant acclimation (*14*). Thus, the purpose of this work was to evaluate the influence of two fertilization regimens and collection date on the organic acids and phenolic compounds profiles of tronchuda cabbage leaves. To accomplish this, a methodology based on HPLC-UV of organic acids was applied to the internal and external leaves aqueous extracts. The internal leaves phenolic compounds were determined by reversed-phase HPLC-DAD analysis of their methanolic extracts.

MATERIALS AND METHODS

Standards and Reagents. Aconitic, citric, ascorbic, and sinapic acids and kaempferol 3-*O*-rutinoside were purchased from Extrasynthése (Genay, France). Malic, shikimic, fumaric, and *p*-coumaric acids were from Sigma (St. Louis, MO). Methanol, sodium hydroxide, and formic acid were purchased from Merck (Darmstadt, Germany), and sulfuric acid was from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

Plant Material and Sampling. Tronchuda cabbages were grown in two fields located in Mirandela, northeastern Portugal (U.T.M. 29 PG5602), according to two different agronomic practices previously described (*12*). Briefly, in one of the fields the production followed the organic status, and in the other field the production was developed according to the standard cultural practices of the region (conventional production). Plant material was sowed by the end of June 2002 and transplanted to the fields at the end of August. In the organic field only organic fertilization was applied with sheep manure. In the conventional field, organic fertilization was made during the transplantation of the plants and, at the beginning of September, a mineral fertilization with ammonium nitrate and CaO (ADP Adubos de Portugal) was applied with a side dress rate of 50 kg of N/ha. In this field, at the end of September, one pesticide treatment with deltamethrin (Decis) (Bayer Crop Science) at a rate of 30 mL/hL was made.

Plant material was harvested for four consecutive months (**Tables 1** and **2**). At each harvesting date and in each field three plants were randomly selected and collected in the morning. After harvesting, the plants were immediately transported to the laboratory and the dark green external leaves were separated from the pale yellow internal ones. Samples were stored in a freezer at −20 °C and then lyophilized (Modulyo 4K Freeze-Dryer Edwards). The three lyophilized materials were powdered, mixed, and kept in an exsiccator, in the dark. Each sample corresponds to a mixture of the external or internal leaves of the three plants, collected in the same field and on the same date.

Organic Acids Extraction. Internal and external leaf extracts were prepared by putting 1.5 g of lyophilized plant material in 300 mL of boiling water. The mixture was boiled for 1 h and then filtered over a Büchner funnel. The resulting extracts were then lyophilized (Modulyo 4K Freeze-Dryer Edwards). The lyophilized extracts were kept in an exsiccator in the dark and redissolved in 0.01 N sulfuric acid prior to analysis by HPLC-UV.

Phenolic Compounds Extraction. Each sample (∼0.5 g) was thoroughly mixed with methanol until complete extraction of the phenolic compounds (negative reaction to 20% NaOH). The extract

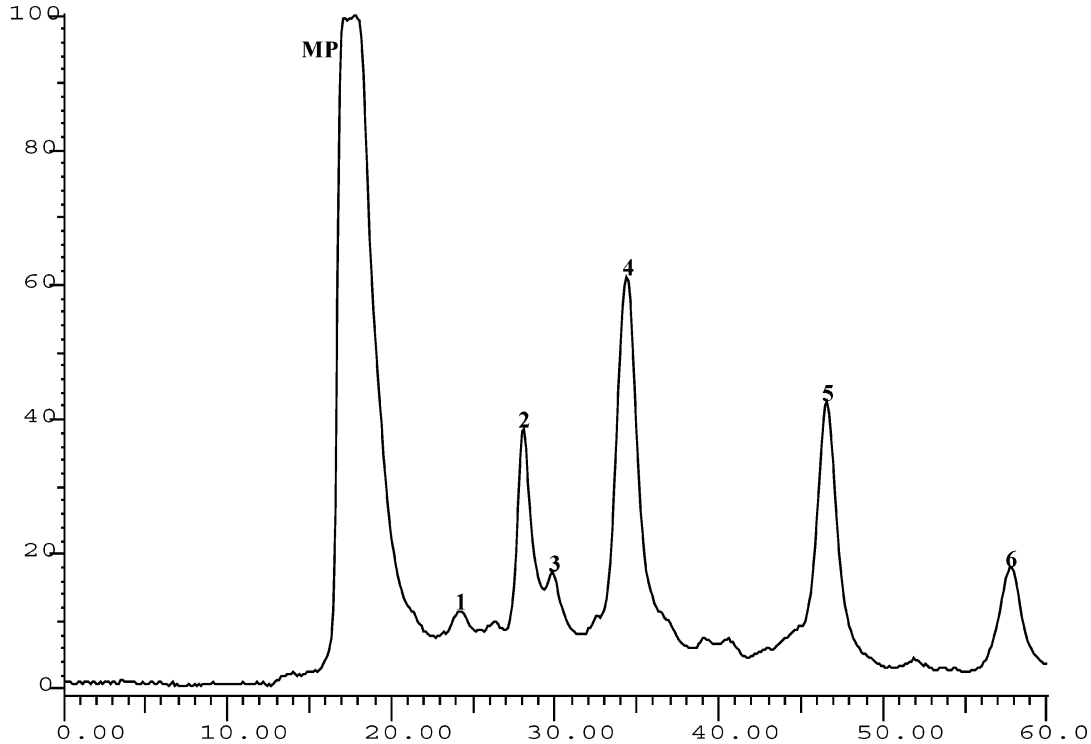

Figure 1. HPLC-UV organic acid profile of tronchuda cabbage internal leaves. Detection was at 214 nm. Peaks: (MP) mobile phase; (1) aconitic acid; (2) citric acid; (3) ascorbic acid; (4) malic acid; (5) shikimic acid; (6) fumaric acid.

was concentrated to dryness under reduced pressure (30 °C), redissolved in methanol (0.5 mL), and 20 μL was analyzed by HPLC-DAD.

HPLC Analysis of Organic Acids. Twenty microliters of each extract was analyzed as previously reported (*9*) with some modifications. The system consisted of an analytical HPLC unit (Gilson) with an ion exclusion column, Nucleogel Ion 300 OA (300 × 7.7 mm), in conjunction with a column-heating device set at 30 °C. Elution was carried out isocratically, at a solvent flow rate of 0.2 mL/min, with 0.01 N sulfuric acid. The injection volume was 20 μL. Detection was performed with a UV detector set at 214 nm.

Organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. The peaks in the chromatograms were integrated using a default baseline construction technique.

HPLC Analysis of Phenolic Compounds. The separation was carried out with a HPLC unit (Gilson) and a 250 × 4.6 mm i.d., 5 μm Spherisorb ODS2 column (Waters, Milford, MA). The solvent system was a mixture of formic acid 5% in water (A) and methanol (B), at a flow rate of 1 mL/min. Elution started with 10% B and reached 20% B at 25 min, 50% B at 40 min, 50% B at 45 min, 90% B at 46 min, and 90% B at 48 min. Detection was achieved with a Gilson diode array detector. Spectroscopic data from all peaks were accumulated in the range of 200−400 nm, and chromatograms were recorded at 330 nm. The data were processed on Unipoint system software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. The peaks in the chromatograms were integrated using a default baseline construction technique. Because standards of the compounds identified in the internal leaf methanolic extracts were not commercially available, 3-*p*-coumaroylquinic acid was quantified as *p*-coumaric acid, the kaempferol derivatives were quantified as kaempferol 3-*O*-rutinoside, and sinapic acid derivatives were quantified as sinapic acid.

RESULTS AND DISCUSSION

Organic Acids. Tronchuda cabbage internal and external leaves presented a chemical profile composed by six identified organic acids: aconitic, citric, ascorbic, malic, shikimic, and fumaric acids (**Figure 1**). The external leaves from organic culture exhibited aconitic acid only in December (**Table 1**).

The lyophilized extracts showed a high content of organic acids, ranging from ca. 11 to 87 g/kg (**Table 1**). Fumaric acid was the compound present in lower amounts, with the exception of sample 10, in which aconitic acid was the minor organic acid (**Table 1**). In the external leaves malic and ascorbic acids were the compounds present in highest amounts, representing from 69 to 93% of total acids, in samples from both organic and conventional culture (**Table 1**). The internal leaves exhibited more variety in the relative amounts of each organic acid. Anyway, in these samples malic acid was the major compound until December, accounting for 43−87% of total identified compounds, and in January ascorbic acid became the main compound, corresponding to 57−69% of total acids (**Table 1**).

With regard to the agronomic procedure both internal and external leaves from organic culture exhibit a similar behavior. The date of collection affects the organic acids profile in the same way, with an increase of ascorbic acid relative amount in January. The highest production of organic acids in the organic samples occurred in December (**Table 1**), following the development of the cabbage: organic tronchuda cabbage presented more developed leaves than those of conventional culture in the same period. This is in accordance with previous results (*12*), in which the commitment of organic tronchuda cabbage cells to morphogenic developmental pathways was accompanied by the lowest level of secondary metabolites (phenolics) in December. Apparently, the nutrients are mainly used for primary metabolites biosynthesis (*15*), which is more related with cabbage growth.

The conventional procedure seems to affect the organic acids profile of tronchuda cabbage, resulting in some discrepancies in the relative amounts of the compounds of external and internal leaves (**Table 1**). In the internal leaves ascorbic acid is a vestigial compound until December (**Table 1**), a fact that remains unexplained. October was the month in which the production

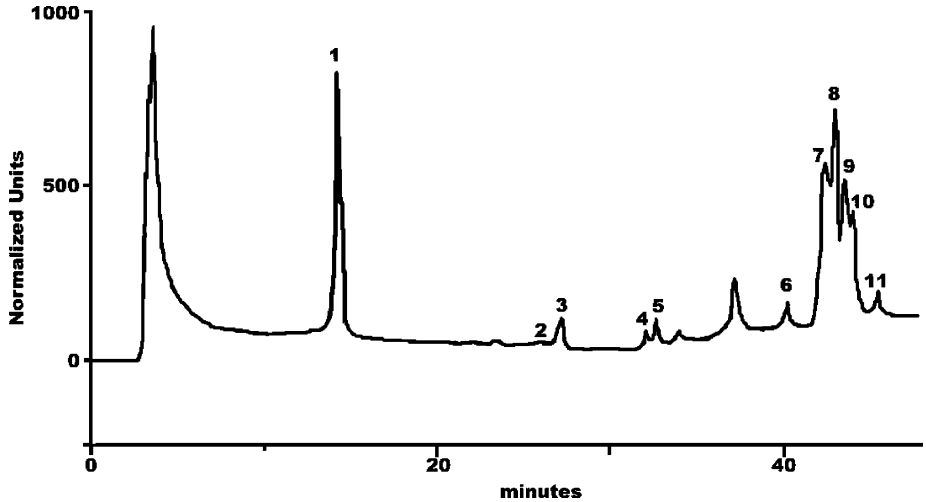

Figure 2. HPLC-DAD phenolic profile of tronchuda cabbage internal leaves. Detection was at 330 nm. Peaks: (1) 3-*p*-coumaroylquinic acid; (2) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (3) kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside; (4) kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside; (5) kaempferol 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside; (6) kaempferol 3-*O*-sophoroside; (7) 1,2-disinapoylgentiobiose; (8) 1-sinapoyl-2-feruloylgentiobiose; (9) isomer of 1,2-disinapoylgentiobiose; (10) 1,2,2′-trisinapoylgentiobiose; (11) 1,2′-disinapoyl-2-feruloylgentiobiose.

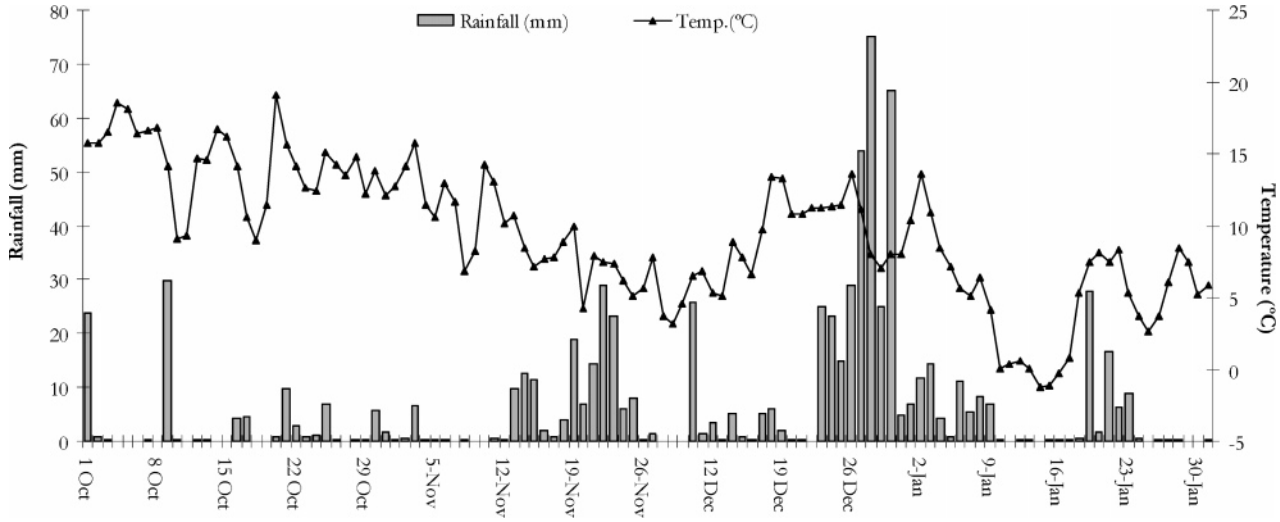

Figure 3. Climatic conditions observed in Mirandela from October 2002 to January 2003.

of citric and malic acids by the samples from conventional practice, subjected to mineral fertilization, was higher. This could be attributed to the existence of nitrate in the fertilizer, which is available in high quantity by that time, leading to higher citric and malic acids contents, as described before (*16*).

Phenolic Compounds. The analysis by HPLC-DAD of the methanolic extracts of tronchuda cabbage internal leaves revealed the presence of several hydroxycinnamic acids and kaempferol derivatives: 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside, two isomers of 1,2-disinapoylgentiobiose, 1-sinapoyl-2-feruloylgentiobiose, 1,2,2′-trisinapoylgentiobiose, and 1,2′-disinapoyl-2-feruloylgentiobiose (**Figure 2**). Kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**2**) was found only in the samples collected in January (samples 15 and 16). The above-mentioned compounds have already been characterized before in tronchuda cabbage internal leaves (*13*). The results obtained in this study indicate that the phenolic composition of tronchuda cabbage internal leaves is distinct from that of the external ones: the internal leaves present phenolic acid derivatives as the main compounds and small amounts of flavonol glycosides (**Figure 2**), whereas external leaves exhibit only flavonol derivatives (*12*). Besides, kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**2**) and kaempferol 3-*O*-sophoroside (**6**) are the only phenolic compounds presented in both kinds of leaves.

Data from the quantification of the identified phenolic compounds (**Table 2**) showed that 3-*p*-coumaroylquinic acid (**1**) and the sinapic acid derivatives, namely, the two isomers of 1,2-disinapoylgentiobiose (**7** and **9**), 1-sinapoyl-2-feruloyl-gentiobiose (**8**) and 1,2,2′-trisinapoylgentiobiose (**10**), were the major compounds, representing >79% of total phenolics, with the exception of sample 16, collected in January from the conventional culture, in which kaempferol 3-*O*-(sinapoyl)-sophoroside-7-*O*-glucoside (**4**) was the compound present in highest amount (∼29% of total phenolics). Compound **11** (1,2′-disinapoyl-2-feruloylgentiobiose) was the minor compound (**Table 2**). The phenolic profile of tronchuda cabbage internal leaves was revealed to be more homogeneous than that of the external ones (*12*), which is not surprising considering that the internal leaves are less exposed to external factors and phenolic compounds are very susceptible to the external environment.

Generally, internal leaf samples from organic culture exhibited higher total phenolics content than those from conventional practice collected in the same period, as was observed with the

external leaves (*12*), with the exception of the samples from November. The interference of the mineral fertilizers and/or pesticides, used in conventional culture, in the biosynthetic pathway of phenolic compounds could explain the lower amounts presented by those samples.

When considering the changes in phenolic composition during winter, we observed a decrease of the total phenolics content until December, which was more evident in samples from conventional culture. A considerable increase of total phenolics in both organic and conventional samples was noticed in January, as was observed before with the external leaves (*12*) (**Table 2**). Additionally, the production of flavonoids was higher in January for the two agronomic practices, a fact that could be explained by the very low temperatures registered in Mirandela region during January (**Figure 3**). In fact, the existence of a positive correlation between high levels of flavonoid glycosides and increased frost resistance is well-known (*17*). As these compounds contain sugar residues, they can delay water crystallization by the formation of hydrogen bonds between their hydroxyl groups and water molecules. Also, the action of flavonoids as antioxidants can be invoked, as protectors of plant tissues against adverse effects of low-temperature oxidative stress (*17*). It seems that phenylalanine ammonia-lyase activity, a key enzyme of phenylpropanoid biosynthesis, is increased under low-temperature conditions (*14*), which may justify the production of flavonoids as defense agents.

The results obtained in this study indicate that, in a general way, tronchuda cabbages from organic culture present higher phenolics contents than those from the conventional one. Tronchuda cabbage may constitute a good source of health-promoting compounds, namely, organic acids and phenolic compounds. It should be emphasized that internal and external leaves supply distinct phenolics. This could be of great relevance when biological activities are considered and deserves further studies.

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4.5. Phenolic compounds in external leaves of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC)

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Phenolic Compounds in External Leaves of Tronchuda Cabbage (*Brassica oleracea* L. var. *costata* DC)

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Glycosylated kaempferol derivatives from the external leaves of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) characterized by reversed-phase HPLC-DAD-MS/MS-ESI were kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside, kaempferol 3-*O*- (methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside, kaempferol 3-*O*-sophoroside-7-*O*-sophoroside, kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside, kaempferol 3-*O*-(sinapoyl/caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside, kaempferol 3-*O*-(sinapoyl)sophoroside, kaempferol 3-*O*-(feruloyl)sophorotrioside, kaempferol 3-*O*-(feruloyl)sophoroside, kaempferol 3-*O*-sophoroside, and kaempferol 3-*O*-glucoside. These acylated derivatives are reported for the first time in nature, with the exception of kaempferol 3-*O*-(sinapoyl)sophoroside. Quantification of the identified compounds was achieved by HPLC-DAD and carried out in samples cultivated under conventional or organic practices and collected at different times. In general, samples from organic production exhibited higher total phenolics content than those from conventional practices collected in the same period.

KEYWORDS: ***Tronchuda cabbage*; *Brassica oleracea* L. var. *costata* DC; kaempferol derivatives; HPLC-DAD-MS/MS-ESI; HPLC-DAD**

INTRODUCTION

Brassica vegetables are consumed in enormous quantities throughout the world and are important in human nutrition. *Brassica oleracea* is a native of the Mediterranean region and southwestern Europe, extending northward to southern England, growing on seaside cliffs. The wild species has evolved into a number of varieties in which different parts of the plant have become the edible constituents. Although essentially temperate, *Brassica oleracea* forms are now grown in other regions all over the world (*1*). Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) is still considered to be a primitive cultivar, being high yielding, less susceptible to pests and diseases, well adapted to a wide range of climates, and generally grown with little or no agrochemical input (*2*, *3*). It is a hardy crop offering the possibility of harvesting in the cold and frosty regions of northern Portugal and Spain, in which it constitutes a very important supply of vegetables during the winter (*2*, *3*). The tronchuda cabbage plant resembles a thick-stemmed collard with large floppy leaves. Leaves are close together, round, smooth, and slightly notched at the margins and are eaten raw or cooked. The internal and external leaves are considerably different with regard to organoleptic characteristics, which may influence the preferences of consumers. Internal leaves are pale yellow and are tender and sweeter than external leaves, which present a dark green color. Previous research on tronchuda cabbage concerned its glucosinolate, protein, mineral, and free sugars composition (*2*−*4*), its resistance to crucifer downy mildew (*5*), the effect of silver nitrate on anther culture embryo production (*6*), and the effect of medium renovation and incubation temperature regimes on microspore culture embryogenesis (*7*).

In the past few years there has been growing interest in the chemopreventive and chemotherapeutic potential of naturally occurring compounds. *Brassica* species are reported to possess cancer preventive properties (*8*) that have been attributed to the glucosinolates and their derived products (*9*). Flavonoids and other phenolics also contribute to this capacity (*10*, *11*). Several studies have reported the presence of polyphenolic compounds in different *B. oleracea* varieties (*12*−*15*), but none involved tronchuda cabbage. Those polyphenols consisted of complex

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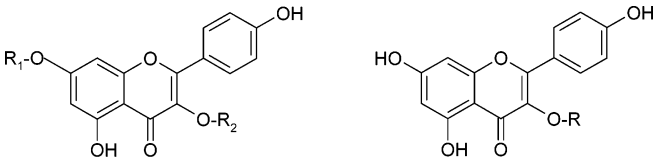
Table 1. Quantification of Tronchuda Cabbage Phenolic Compounds (Milligrams per Kilogram, Dry Basis)[a]

sample	collection date	culti-vation[c]	compound[b]										total
			1 + 2	**3**	**4**	**5 + 6**	**7**	**8**	**9 + 10**	**11**	**12 + 13**	**14**	
1	Oct 2002	O	556.9 (36.8)	2.4 (0.1)	5.8 (0.1)	4.2 (0.0)	91.6 (5.9)	45.5 (2.6)	157.6 (10.2)	15.4 (0.4)	87.8 (1.8)	nq[d]	912.3
2	Oct 2002	C	274.4 (11.2)	112.6 (2.8)	12.6 (0.5)	10.5 (0.8)	22.3 (1.6)	95.4 (2.4)	59.2 (1.9)	6.7 (0.8)	45.8 (1.8)	nq	555.8
3	Nov 2002	O	547.6 (46.0)	750.2 (84.0)	21.3 (2.5)	59.1 (15.4)	186.5 (10.7)	429.7 (42.2)	173.1 (6.7)	16.2 (1.9)	240.1 (29.6)	nq	1952.0
4	Nov 2002	C	191.0 (16.3)	16.0 (9.3)	8.1 (0.1)	18.2 (0.3)	1.3 (0.1)	96.7 (14.3)	97.1 (8.9)	100.1 (1.5)	38.7 (3.0)	nq	561.4
5	Dec 2002	O	9.2 (0.7)	8.6 (0.1)		3.3 (0.1)	1.3 (0.0)	1.6 (0.2)	2.0 (0.2)	1.1 (0.1)	7.5 (0.2)	nq	27.4
6	Dec 2002	C	18.4 (0.7)	41.9 (0.6)	3.3 (0.1)	3.8 (0.2)	7.3 (0.1)	9.3 (3.4)	60.5 (2.7)	94.7 (4.6)	23.0 (0.0)	nq	252.5
7	Jan 2003	O	245.7 (22.1)	53.9 (2.9)	2.5 (0.0)	80.4 (1.9)	5.9 (0.1)	32.3 (2.5)	115.7 (2.6)	185.7 (3.4)	3.2 (0.1)	nq	712.6
8	Jan 2003	C	62.5 (1.3)	8.0 (0.6)	1.4 (0.1)	3.5 (0.5)	34.2 (0.2)	2.1 (0.0)	34.5 (1.1)		33.6 (2.7)	nq	254.1

[a] Results are expressed as mean (standard deviation) of two determinations. [b] Identity of compounds as in **Figure 1**. [c] O, organic; C, conventional [d] nq, not quantified.

flavonol glycosides, some of them being acylated derivatives, and hydrocycinnamic acid esters.

In a preliminary study we observed marked differences between the phenolic compositions of the two kinds of leaves. The work herein constitutes the first step for the characterization of phenolics in this cabbage. We aimed to identify and quantify the phenolics from tronchuda cabbage external leaves and to see if the phenolic composition is influenced by the agricultural procedure and the collection date. For this purpose, a methodology based on the solid-phase extraction (SPE) of phenolic compounds and reversed-phase HPLC-DAD-MS/MS-ESI and HPLC-DAD analysis of the methanolic extracts was applied to external leaf samples.



	R_1	R_2		R
1	glc	sophtr	**9**	sophtr
2	glc	soph-methoxycaffeic/caffeic	**10**	soph-sinapic
3	glc	soph	**11**	sophtr-ferulic
4	soph	sophtr	**12**	soph-ferulic
5	soph	soph	**13**	soph
6	soph	glc-glc-glc-glc	**14**	glc
7	glc	soph-sinapic/caffeic		
8	glc	soph-ferulic/caffeic		

Figure 1. Chemical structures of phenolic compounds identified in tronchuda cabbage: glc, glucose; soph, sophorose; sophtr, sophorotriose.

MATERIALS AND METHODS

Standards and Reagents. Kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucoside were from Extrasynthése (Genay, France). Methanol, sodium hydroxide, and hydrochloric and formic acids were purchased from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

SPE Columns. The Isolute C18 non-endcapped (NEC) columns (50 μm particle size, 60 Å porosity; 10 g of sorbent mass/70 mL of reservoir volume) were obtained from International Sorbent Technology Ltd. (Mid Glamorgan, U.K.).

Plant Material and Sampling. Tronchuda cabbages grown under different agronomic practices were studied. The experimental work was carried out in two fields located in Valbom dos Figos−Mirandela, northeastern Portugal (U.T. M. 29 PG5602). In one of the fields the production has organic status, certified by the national authority (Instituto de Desenvolvimento Rural e Hidráulica), following the guidelines of Council Regulation (EEC) 2092/91 of June 24, 1991 (organic production), and in the other field the production followed the standard cultural practices in the region (conventional production) (**Table 1**). The sowing date was in the end of June 2002, and the plant material was transplanted to the fields in the end of August. Water was provided by a local captation. In the organic field only organic fertilization was made with sheep manure, and no phytosanitary treatments were applied. In the conventional field, organic fertilization was made during the transplantation of the plants and, in the beginning of September, a mineral fertilization with 20.5% ammonium nitrate and 21.8% CaO (Nitrolusal 20.5%) (ADP Adubos de Portugal) was applied with a side dress rate of 50 kg of N/ha. In this field, at the end of September, one pesticide treatment with deltamethrin (Decis) (Bayer Crop Science) at a rate of 30 mL/hL was made.

Plant material was harvested for four months (**Table 1**). On each harvesting date and in each field three plants were randomly selected and collected in the morning, approximately at the same hour. After harvesting, the plants were immediately transported to the laboratory and external and internal leaves were separated. Each sample corresponds to a mixture of the external leaves of the three plants, collected in the same field. Samples were stored in a freezer and freeze-dried before analysis. External leaves were subjected to phenolics extraction and HPLC analysis.

Phenolic Compound Extraction. Each sample (.∼0.5 g) was thoroughly mixed with methanol until complete extraction of the phenolic compounds (negative reaction to 20% NaOH). The extract was concentrated to dryness under reduced pressure (30 °C) and redissolved in water acidified to pH 2 with HCl. The solution obtained was applied to an Isolute C18 (NEC) column, previously conditioned with 30 mL of methanol and 70 mL of acidified water. Polar compounds were removed with the aqueous solvent, and the retained phenolic compounds were then eluted with 50 mL of methanol. The extract was concentrated to dryness under reduced pressure (30 °C) and redissolved in methanol (1 mL).

Alkaline and Acid Hydrolysis. Hydrolysis was achieved according to previously reported methodology (*12*) by adding 0.5 mL of 4 N NaOH to the methanolic extract (0.5 mL) and keeping the mixture for 16 h at room temperature in a stoppered test tube under N_2 atmosphere. After this step, the alkaline hydrolysis products were acidified with concentrated HCl to pH 1−2 and directly analyzed by LC/UV-DAD/ESI-MS^n. This solution was used for partial (mild) acid hydrolysis and kept in a stoppered test tube during 30 min in an oven adjusted to 80 °C; then, it was directly analyzed by HPLC-DAD-MS/MS.

Total acid hydrolysis was carried out by adding 0.5 mL of 4 N HCl to 0.5 mL of the methanolic extract, and this solution was kept in a stoppered test tube, incubated for 30 min at 85 °C, and directly analyzed by LC/UV-DAD/ESI-MS^n.

HPLC-DAD-MS/MS-ESI Qualitative Analysis. Chromatographic separations were carried out on a 250 × 4 mm i.d., 5 μm, RP-18 LiChroCART column (Merck, Darmstadt, Germany) protected with a 4 × 4 mm LiChroCART guard column using formic acid 0.1% (A) and methanol (B) as solvents, starting with 20% B and using a gradient to obtain 50% B at 35 min and 80% B at 37 min. On the other hand, for the analysis of the acids and the aglycons obtained after hydrolysis, a linear gradient starting with 15% B and reaching 65% B at 50 min was used to reach 80% B at 52 min. The flow rate was 1 mL/min, and the injection volumes varied between 10 and 50 μL.

The HPLC system was equipped with an Agilent 1100 series diode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser, and a G1315B photodiode array detector controlled by ChemStation software (v. 08.03). Spectroscopic data from all peaks were accumulated in the range 240−400

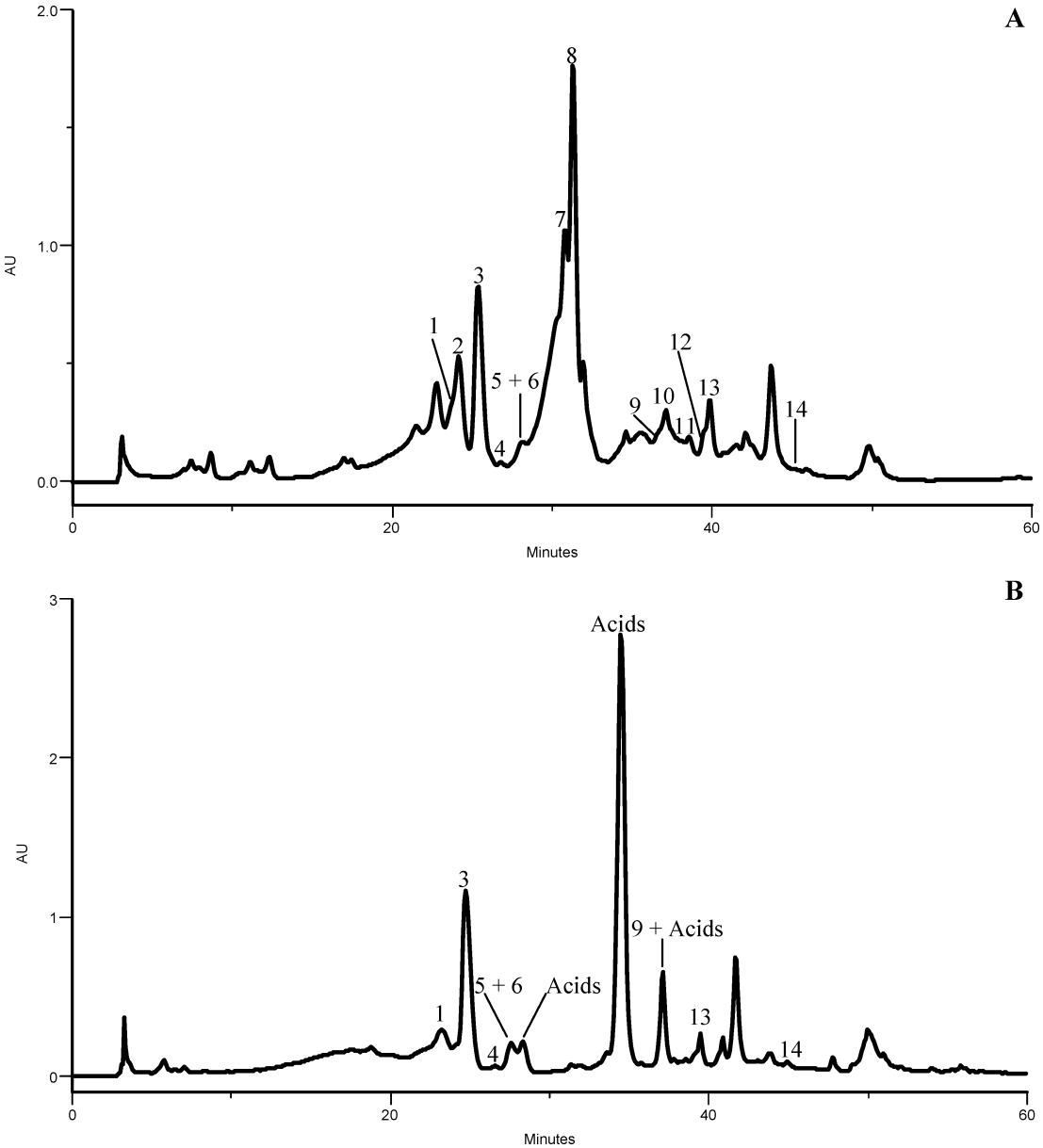


Figure 2. (**A**) HPLC-DAD chromatogram of tronchuda cabbage methanolic extract. (**B**) HPLC-DAD chromatogram of tronchuda cabbage methanolic extract after alkaline hydrolysis: (peak 1) kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside; (peak 2) kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside; (peak 3) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (peak 4) kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside; (peak 5) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside; (peak 6) kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside; (peak 7) kaempferol 3-*O*-(sinapoyl/caffeoyl)sophoroside-7-*O*-glucoside; (peak 8) kaempferol 3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside; (peak 9) kaempferol 3-*O*-sophorotrioside; (peak 10) kaempferol 3-*O*-(sinapoyl)sophoroside; (peak 11) kaempferol 3-*O*-(feruloyl)sophorotrioside; (peak 12) kaempferol 3-*O*-(feruloyl)sophoroside; (peak 13) kaempferol 3-*O*-sophoroside; (peak 14) kaempferol 3-*O*-glucoside. Detection at 330 nm.

nm, and chromatograms were recorded at 330 nm for glycosides and acylated derivatives and at 330 and 360 nm for hydroxycinnamic acids and flavonoid aglycons, respectively. The mass detector was an Agilent G2445A ion trap mass spectrometer equipped with an electrospray ionization (ESI) system and controlled by LCMSD software (v. 4.1). Nitrogen was used as nebulizing gas at a pressure of 65 psi, and the flow was adjusted to 11 L/min. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full scan mass covered the range from *m*/*z* 200 to 2000 for free glycosides and acylated derivatives and from *m*/*z* 90 to 400 for acids and aglycons. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 to 2 V. MS data were acquired in the negative ionization mode. MS^n data were achieved in the automatic mode on the more abundant fragment ion in MS^{n-1}. **Tables 2** and **3** show the most frequent ions that characterize the fragmentation of the flavonoid *O*-glycosides. Other ions were found, but they have not been included due to their low significance on the MS behavior ions.

HPLC-DAD Quantitative Analysis. Twenty microliters of each extract was analyzed using a HPLC unit (Gilson) and a 250 × 4.6 mm i.d., 5 μm Spherisorb ODS2 column (Waters, Milford, USA). The solvent system was a mixture of formic acid 5% in water (A) and methanol (B), with a flow rate of 1 mL/min, and the gradient was as follows: 0 min, 10% B; 25 min, 20% B; 40 min, 50% B; 45 min, 50% B; 46 min, 90% B; 50 min, 90% B; 55 min, 100% B; 58 min, 100% B; and 60 min, 10% B. Detection was achieved with a Gilson diode array detector. Spectroscopic data from all peaks were accumulated in the range of 200−400 nm, and chromatograms were recorded at 330 nm. The data were processed on Unipoint system software (Gilson

Table 2. t_R, -MS $[M - H]^-$, -MS^2 $[M - H]^-$, and -MS^3 $[(M - H) \rightarrow Y^7{}_0]^-$ Data of Kaempferol Glycosides

compound[a]	t_R (min)	$[M - H]^-$ (*m/z*)	-MS^2 $[M - H]^-$ (*m/z*) (%) $Y^7{}_0{}^-$	-MS^3$[(M - H) \rightarrow Y^7{}_0]^-$ (*m/z*) (%) $Y^7{}_0{}^{0,2}X^-$	$Y^7{}_0Y^3{}_3{}^-$	$Y^7{}_0Y^3{}_2{}^-$	$Y^7{}_0Y^3{}_1{}^-$	$Y^7{}_0Z^3{}_1{}^-$	$Y^7{}_0Y^3{}_0{}^-$
Kaempferol-3-*O*-tetraglcucoside-7-*O*-sophoroside									
			(–324)		(–162)	(–324)			(–648)
6, kaempf-3-TetraGlc-7-Soph	10.8	1257	933 (100)		771 (12)	609 (35)			285 (100)
Kaempferol-3-*O*-sophorotrioside-7-*O*-glucoside/sophoroside									
			(–162/–324)	(–120)		(–162)		(–342)	(–486)
1, kaempf-3-Sophtr-7-Glc	9.1	933	771 (100)			609 (100)		429 (72)	285 (39)
4, kaempf-3-Sophtr-7-Soph	10.2	1095	771 (100)	651 (6)		609 (100)		429 (35)	285 (71)
Kaempferol-3-*O*-sophoroside-7-*O*-glucoside/sophoroside									
			(–162/–324)	(–120)			(–162)	(–180)	(–324)
3, kaempf-3-Soph-7-Glc	10.0	771	609 (100)				447 (17)	429 (45)	285 (100)
5, kaempf-3-Soph-7-Soph	10.5	933	609 (100)	489 (11)				429 (92)	284 (100)[b]

Kaempferol-3-*O*-glycoside

compound[a]	t_R (min)	$[M - H]^-$ (*m/z*)	-MS^2 $[M - H]^-$ (*m/z*) (%) $^{0,2}X^-$	$Y^3{}_2{}^-$	$Z^3{}_2$	$Y^3{}_1{}^-$	$Z^3{}_1{}^-$	$Y^3{}_0{}^-$
			(–120)	(–162)	(–180)	(–324)	(–342)	(–486)
9, kaempf-3-Sophtr	22.2	771		609 (27)	591 (29)		429 (63)	285 (100)
						(–162)	(–180)	(–324)
13, kaempf-3-Soph	24.6	609	489			447 (7)	429 (43)	285 (100)
14, kaempf-3-Glc	32.5	447						285 (100)

[a] Kaempf, kaempferol; Glc, glucose; Soph, sophorose; Sophtr, sophorotriose. [b] Fragments from homolytic cleavage of the glycosidic bond ($[Y^7{}_0Y^3{}_0–H]^{\bullet-}$) (*18*).

Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. With the exception of kaempferol 3-*O*-glucoside, which was quantified as itself, the identified compounds were quantified as kaempferol 3-*O*-rutinoside, because none of them was commercially available. The average regression equations for kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucoside were $y = 2.29 \times 10^7x$ and $y = 2.45 \times 10^7x$, respectively. The detection limits were 0.345 μg/mL for kaempferol 3-*O*-rutinoside and 0.322 for kaempferol 3-*O*-glucoside.

RESULTS AND DISCUSSION

Characterization of Phenolic Compounds. The HPLC-DAD chromatogram of tronchuda cabbage methanolic extract revealed the existence of several compounds with UV spectra with two maxima at 267 and 349 nm and a shoulder at 300 nm, indicating the presence of kaempferol derivatives with substitution in position 3 (**Figures 1** and **2A**). In addition, acylated flavonoids were detected in the extract, and their UV spectra, characterized with a maximum with a high absorption at 330 nm and a little maximum between 255 and 268 nm, suggested that they were acylated with hydroxycinnamic acid derivatives. After alkaline hydrolysis, the chromatogram showed, apart from several hydroxycinnamic acids, various flavonoid glycosides and disappearance of the acylated derivatives (**Figure 2B**).

Flavonoid Glycosides. The MS study of free (nonacylated) glycosides was performed on the saponified extract so that the possible glucosyl radical losses (−162 amu) were not confused with those of the caffeoyl radical (−162 amu). The HPLC-DAD chromatogram of the saponified extract exhibited compound **3** as the main deacylated glycoside (**Figure 2B**).

The MS ion trap analysis of compound **3** and other minor flavonoids eluting in the first part of the chromatogram (**1**, **4**, **5**, and **6**), indicated that these compounds were kaempferol hexosides (*m/z* of the aglycon at 285), most probably glucosides due to phylogenetic similarity with cauliflower (*12*) and broccoli (*15*). According to previous studies (*16*), from the study of their fragmentation in MS^n ($n = 2-4$) (**Table 2**) it can be deduced that these derivatives are glycosylated in positions 3 and 7, with glucose, sophorose, or sophorotriose. The fragmentation pattern and the relative abundance of the obtained ions indicated the nature of the oligosaccharides linked to the hydroxyl groups in positions 3 and 7. The partial acid hydrolysis of the raw extract and its subsequent HPLC-DAD-MS^n study gave rise to the characterization, besides kaempferol as the main flavonoid, of kaempferol 7-*O*-glucoside [UV, λ 253sh, 266, 321sh, 367 nm; MS, 447 $(M - H)^-$; MS^2 $(M - H)^-$ 285], confirming the substitution of the hydroxyl at the 7-position of the most abundant glycoside (**3**). According to the above, the following kaempferol derivatives have been characterized both in the raw and in the saponified extracts: kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside (**1**), kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**3**), kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside (**4**), and kaempferol 3-*O*-sophoroside-7-*O*-sophoroside (**5**) (**Figure 1**).

Compounds **9**, **13**, and **14** exhibited UV spectra indicating a kaempferol derivative, with a substituent in position 3. Their fragmentation was characteristic of glycosides linked to only one hydroxyl group (*16*). They were identified as kaempferol 3-*O*-sophorotrioside (**9**), kaempferol 3-*O*-sophoroside (**13**), and kaempferol 3-*O*-glucoside (**14**), respectively. These glycosides have been recently characterized in cauliflower (*12*), some of them being detected for the first time in nature. It should be emphasized that flavonoids with more than three sugar residues are not usual and that pentaglycosides were observed for the first time in cauliflower (*12*) and broccoli (*15*).

The production of flavonoids with an unusually high degree of glycosylation by other *B. oleracea* varieties (*12*, *15*) and the useful application of HPLC-DAD-MS^n ion trap in the identification of these kinds of complex flavonoids (*16*) led us to search for the possible existence of other kaempferol derivatives with six hexoses. Near compound **5** we detected in the extracted ion chromatogram (EIC) an ion at *m/z* 1257 (compound **6**), present in trace amounts, which was in accordance with the deprotonated

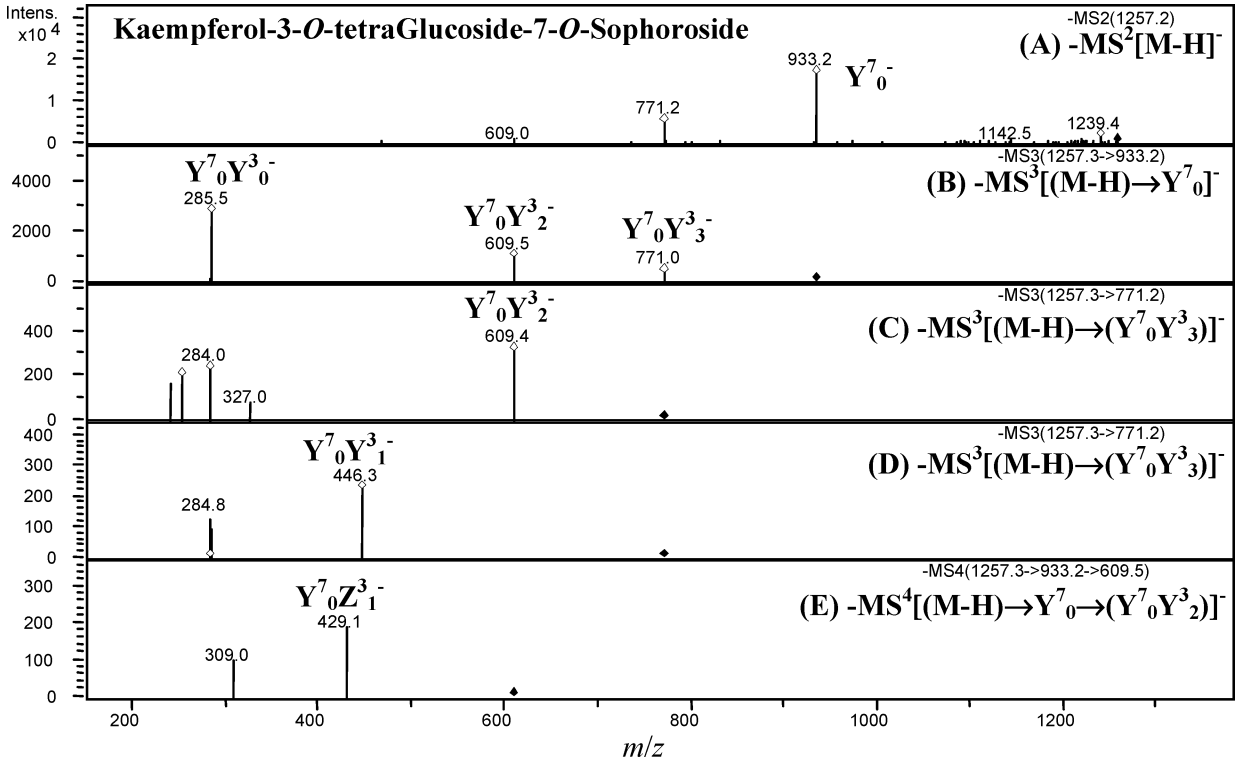


Figure 3. MS^n analysis of kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside (**6**): (**A**) -MS^2 [M – H]$^-$; (**B**) -MS^3 [(M – H) → $Y^7{}_0$]$^-$; (**C**, **D**) -MS^3 [(M – H) → ($Y^7{}_0Y^3{}_3$)]$^-$; (**E**) -MS^4 [(M – H) → $Y^7{}_0$ → ($Y^7{}_0Y^3{}_2$)]$^-$.

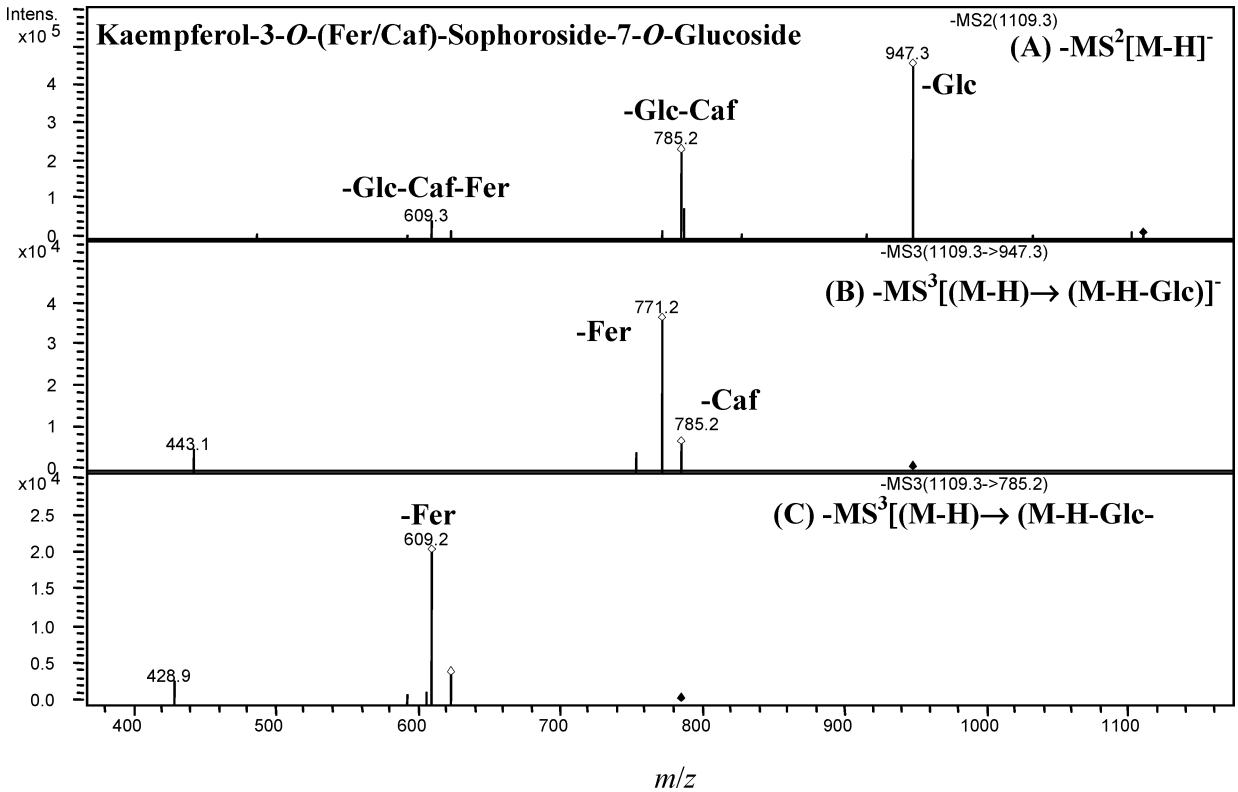


Figure 4. MS^n analysis of kaempferol3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside (**8**): (**A**) -MS^2 [M – H]$^-$; (**B**) -MS^3 [(M – H) → (M – H – Glc)]$^-$; (**C**) -MS^3 [(M – H) → (M – H – Glc – Caf)]$^-$.

molecular ion of an hexaglucosyl kaempferol. The loss of 324 amu, corresponding to two sugar moieties, in the MS^2 (M – H)$^-$ event to give an ion at *m/z* 933 (base peak), $Y^7{}_0{}^-$, indicated the presence of sophoroside linked to the hydroxyl group at the 7-position of kaempferol (**Table 2**; **Figure 3A**) and, therefore, the remaining four sugar residues should be tentatively considered as a tetrasaccharide linked to the hydroxyl group at the 3-position. In addition, the MS^3 [(M – H) → $Y^7{}_0$]$^-$ analysis allowed us to observe, apart from the ion of the aglycon (*m/z* 285), the fragmentation ions at *m/z* 771 and 609, corresponding to the losses of one and two glucoses from the ion at *m/z* 933 (**Figure 3B**), respectively, confirming the proposed substitution.

Other observed ions, which are not included in **Table 2**, were $Y^7{}_0Y^3{}_1{}^-$ (*m/z* 446) and $Y^7{}_0Z^3{}_1{}^-$ (*m/z* 429) (**Figure 3D**,**E**). The ions obtained have been labeled according to previous studies (*16*−*18*). According to the above-mentioned, compound **6** was identified as kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside.

Acylated Derivatives. The comparison of the HPLC-DAD chromatogram of the raw methanolic extract (**Figure 2A**) with that of the saponified extract (**Figure 2B**) indicated the existence of an acylated compound (compound **8**) present in high amounts. The HPLC-MS analysis showed the presence of other acylated compounds (compounds **2**, **7**, **10**−**12**) coeluting with other substances. From the MS study of these compounds two groups of acylated derivatives could be observed. For their retention times, and by comparison with broccoli compounds (*15*), one of them, grouping compounds **2**, **7**, and **8**, should be composed of monoacylated derivatives of kaempferol tetraglycoside, acylated with methoxycaffeic, sinapic, and ferulic acids, respectively. However, once the main product in the saponified extract is kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**3**), they were tentatively considered as being diacylated derivatives of compound **3**, with caffeic acid as the second acylation acid, with a kind of link between the acyl groups that modifies their retention times, without discarding the possibility of being monocylated derivatives of compound **1** (kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside). The other group of compounds (**10**−**12**) were monoacylated derivatives of kaempferol 3-*O*-glycosides, without glycosylation at the 7-position (**Table 3**).

As previously reported (*15*), in the MS^2 $(M - H)^-$ of compounds with glycosylation at both the 3- and 7-positions, a base peak corresponding to the loss of glycosylation at the 7-position was observed. Other important ions were also detected and were due to the simultaneous loss of the mentioned sugar and of one or two acids (**Table 3**; **Figure 4A**). After the base peak (M − H − Glc), the most abundant ion was (M − H − 324), resulting from the simultaneous loss of the glucose at the 7-position and of one caffeic acid (**Table 3**). In the fragmentation of the ion resulting from the loss of glycosylation at the 7-position (MS^3 $[(M - H) \rightarrow (M - H - Glc)]^-$) the loss of the two acids could be observed, confirming that the acylation was present on the sugars at the 3-position (**Table 3**; **Figure 4B**). The MS^3 $[(M - H) \rightarrow (M - H - Glc - Caf)]^-$ event gave rise to the ion of the aglycon glycosylated at the 3-position (kaempferol 3-*O*-sophorosyl) by loss of the other acid (**Table 3**; **Figure 4C**), the breakdown of which (data not shown in **Table 3**) was like the corresponding one for compound **3**. In the MS^3 $[(M - H) \rightarrow (M - H - Glc - Caf)]^-$ event of the derivatives with ferulic or sinapic acid from the first group and in the MS^2 $(M - H)^-$ event of the second group, an ion at *m/z* 623, corresponding to 609 + 14, could also be observed (data not shown in **Table 3**).

Therefore, the following diacyl derivatives from compound **3** have been characterized: kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside (**2**), kaempferol 3-*O*-(sinapoyl/caffeoyl)sophoroside-7-*O*-glucoside (**7**), and kaempferol 3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside (**8**). In addition, the monoacylated derivatives kaempferol 3-*O*-(sinapoyl)-sophoroside (**10**), kaempferol 3-*O*-(feruloyl)sophorotrioside (**11**), and kaempferol 3-*O*-(feruloyl)sophoroside (**12**) have also been identified. To the best of our knowledge, the characterization of these acylated derivatives has not been previously reported in nature, with the exception of compound **10**, recently described in cauliflower (*12*).

The comparative study of tronchuda cabbage flavonoids with those from cauliflower and broccoli indicates their resemblance

Table 3. t_R, -MS $[M - H]^-$, -MS^2 $[M - H]^-$, -MS^3 $[(M - H) \rightarrow (M - H - Glc)]^-$, and -$MS^3$ $[(M - H) \rightarrow (M - H - Glc - Caf)]^-$ Data of Acylated Derivatives from Kaempferol Glycosides

compound[a]	t_R (min)	$[M - H]^-$ (*m/z*)	-$MS^2[M - H]^-$ (*m/z*) (%)								-MS^3 $[(M - H) \rightarrow (M - H - Glc)]^-$ (*m/z*) (%)				-MS^3 $[(M - H) \rightarrow (M - H - Glc - Caf)]^-$ (*m/z*) (%)		
			−162 −Glc	−176 −Fer	−206 −Sinp	−324 −G-C	−338 −G-F	−354 −G-MC	−516 −G-C-MC	−500 −G-C-F	−530 −G-C-S	−162 −Caf	−176 −Fer	−192 −MeOCaf	−206 −Sinp	−176 −Fer	−192 −MeOCaf	−206 −Sinp
						diacylated derivatives from **3**: kaempferol-3-*O*-sophoroside-7-*O*-glucoside											
2, 3-MeOCaf/Caf	9.3	1125	963 (100)			801 (67)		771 (16)	609 (7)			801 (6)		771 (100)			609 (100)
7, 3-Sinp/Caf	12.5	1139	977 (100)			815 (35)				609 (5)		815 (9)			771 (100)		
8, 3-Fer/Caf	13.2	1109	947 (100)			785 (49)	771 (4)			609 (9)		785 (16)	771 (100)			609 (100)		609 (100)
			monoacylated derivatives from kaempferol-3-*O*-glycosides														
10, 13-Sinp	22.6	815			609 (100)												
11, 9-Fer	24.0	947		771 (100)													
12, 13-Fer	24.6	785		609 (100)													

[a] G (Glc), glucosyl; F (Fer), feruloyl; S (Sinp), sinapoyl; MC (MeOCaf), methoxycaffeoyl; **3**, kaempferol-3-*O*-sophoroside-7-*O*-glucoside; **9**, kaempferol-3-*O*-sophorotrioside; **13**, kaempferol-3-*O*-sophoroside.

to the presence of highly glycosylated cinnamoyl derivatives of flavonols.

Quantitative Analysis. The results obtained with the quantification of the identified phenolic compounds (**Table 1**) show that, in general, all 14 identified compounds were detected in the analyzed samples, with the exceptions of sample 5, which did not show kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside (**4**), and sample 8, which did not exhibit kaempferol 3-*O*-(feruloyl)sophorotrioside (**11**).

The highest amounts were found for the pair kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside (**1**) plus kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside (**2**), except for sample 3, in which kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**3**) was the major compound, and sample 6, which showed kaempferol 3-*O*-(feruloyl)sophorotrioside (**11**) as the major constituent. In general, kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside (**4**) and kaempferol 3-*O*-(feruloyl)-sophorotrioside (**11**) were minor compounds, although in sample 6, kaempferol 3-*O*-(feruloyl)sophorotrioside (**11**) is the compound present in highest amounts, as referred before. Kaempferol 3-*O*-glucoside (**14**) exists in trace amounts. Generally, samples from organic culture exhibited higher total phenolics content than those from conventional practices, collected in the same period, with the exception of the samples from December. This might be due to the interference of the mineral fertilizers and/or pesticides, used in conventional practices, in the biosinthetic pathway of flavonoids, decreasing phenolic amounts.

When considering the evolution during winter, we observed that between November and December there was a decrease of the total phenolics content, which is more evident in samples from organic culture. Additionally, a considerable increase of total phenolics in organic samples was noticed in January, mainly due to a higher content of the pairs kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside (**1**) plus kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside (**2**), kaempferol 3-*O*-sophoroside-7-*O*-sophoroside (**5**) plus kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside (**6**) and kaempferol 3-*O*-sophorotrioside (**9**) plus kaempferol 3-*O*-(sinapoyl)sophoroside (**10**) and of kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**3**), kaempferol 3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside (**8**), and kaempferol 3-*O*-(feruloyl)sophorotrioside (**11**). Geographical and climatic conditions cannot justify these observations, because all of the samples were collected in the same area. The lowest level of total phenolics observed in December in sample from organic production may be explained by the commitment of tronchuda cabbage cells to morphogenic developmental pathways, because those cabbages presented more developed leaves than those of conventional culture in the same period. Apparently that effect is less compatible with the biosynthesis and or turnover of phenolic secondary metabolites (*19*, *20*).

The results obtained in this study suggest that tronchuda cabbage external leaves may constitute a good source of health-promoting compounds, namely, flavonoids. However, more tronchuda cabbage external leaves, from other geographical origins, should be analyzed to establish the factors that affect its phenolic quantitative profile.

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4.6. Multivariate analysis of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) phenolics: Influence of fertilizers

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Multivariate Analysis of Tronchuda Cabbage (*Brassica oleracea* L. var. *costata* DC) Phenolics: Influence of Fertilizers

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A field experiment was carried out to investigate the effect of fertilization level on the phenolic composition of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) external and internal leaves. Eight different plots were constituted: a control without fertilization, one with organic matter, and six experiments with conventional fertilizers (nitrogen, boron, and sulfur, two levels each). The phenolic compounds were analyzed by reversed-phase HPLC-DAD. External and internal leaves revealed distinct qualitative composition. In the internal leaves were found 15 phenolics (5 kaempferol and 10 cinnamic acid derivatives), whereas the external leaves presented 3-*p*-coumaroylquinic acid and 13 kaempferol derivatives. Principal component analysis (PCA) was applied to assess the relationships between phenolic compounds, agronomical practices, and harvesting time. Samples obtained with conventional practices were quite effectively separated from organic samples, for both types of leaves. In general, samples developed without any fertilization presented the highest phenolics amounts: external and internal leaves contained 1.4- and 4.6-fold more phenolic compounds than the ones that received conventional fertilizer, respectively, and the internal leaves presented 2.4 times more phenolics than the ones grown with organic amendment. Additionally, samples from organic production exhibited higher total phenolics content than those from conventional practices, collected at the same time. Samples harvested first were revealed to be distinct from the ones collected later. The results show that it is possible to grow tronchuda cabbage without excess fertilizers, with highest amounts of phenolics and reduced environment contamination.

KEYWORDS: ***Brassica oleracea* L. var. *costata* DC; tronchuda cabbage; phenolic compounds; organic fertilization; nitrogen; boron; sulfur; principal component analysis**

INTRODUCTION

It is well established that a greater daily intake of vegetables and fruits is associated with a smaller risk of the major deadly diseases in Western society. Plant secondary metabolites are the most likely candidates for this general health-promoting effect (*1*). Epidemiological studies indicate that consumption of cruciferous vegetables is more strongly associated with decreased cancer risk than fruit and vegetable consumption in general, which can be attributed to the presence of sulfur-containing compounds (*2*). Other phytochemicals, such as phenolic compounds, may also contribute to this effect.

Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC), mainly consumed for its leaves, has already been characterized in terms of glucosinolates (*3, 4*) and phenolics (*5, 6*). Although genetics and seasonally induced changes are the primary determinants of the composition of secondary plant metabolites, the phenolics content may also be affected by environmental factors and the fertilization regimen (*7, 8*).

Soil mineral nitrogen availability may vary according to the amount of applied nitrogen and crop nitrogen uptake pattern (*9*). Nitrogen uptake, which is genetically determined, in Brassicas is higher than in many other food crops. Despite this,

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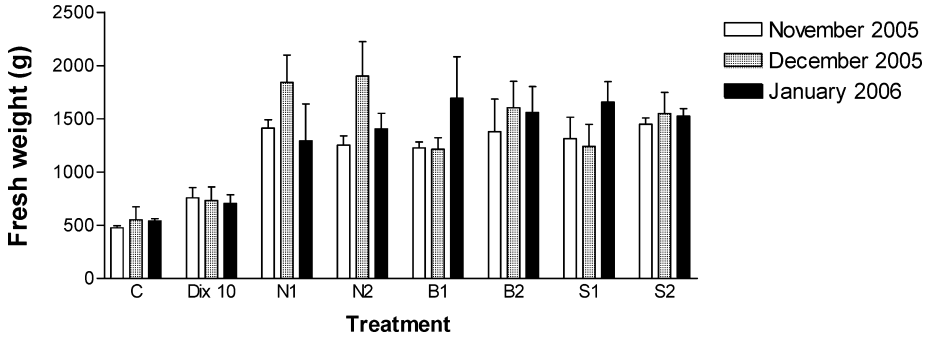

Figure 1. Fresh weight of *B. oleracea* var. *costata* plants subjected to different fertilization regimens. Results are expressed as means of three replicates, and standard deviation bars are on top of each column. C, control; Dix10, organic amendment; N1, 80 kg of N/ha; N2, 160 kg of N/ha; B1, 2.2 kg of B/ha; B2, 4.4 kg of B/ha; S1, 37.3 kg of S/ha; S2, 74.6 kg of S/ha.

a significant part of the nitrogen taken up is lost to the soil in dead leaves, during the growth cycle (*10*).

In contrast to conventional systems, in which synthetic fertilizers containing directly available inorganic nitrogen are used, organic systems rely on the activity of a diverse soil ecosystem to make nitrogen available to plants (*11, 12*). It has been shown that nitrogen can become a growth-limiting nutrient in organic production, affecting negatively yield (*12*). This problem is overcome by conventional farmers, who obtain roughly the same nutrient supply across a variety of soil types and farming systems, because fertilization is adjusted to the level that gives the highest yield. On the other hand, conventional farming systems are often associated with problems such as nitrate leaching and groundwater pollution, degradation of soil structure, decreased surface infiltration of water, and pesticide contamination (*9, 13*). In fact, the lower nitrogen mineralization rate in organic farming system may improve nutrient recycling and reduce the risk for nitrogen leaching and groundwater pollution (*1, 9*).

The production of phenolic metabolites responds to changes in nutrient availability in a highly complex manner (*14*). Nitrogen stress triggers the gene expression of flavonoid pathway enzymes, and nitrate availability was shown to directly affect the enzyme activity in the phenylpropanoid pathway (*7, 15–17*). Some authors propose that variation in concentrations of secondary metabolites may be due to differences in plant growth and accumulation of biomass (*14*). Others noticed that some phenolics, such as kaempherol and quercetin, do not seem to be affected by nutrition levels to the same extent as plant growth (*16, 18*).

Boron is required for normal growth and development of all higher plants, and it is thought to be involved in three main aspects of plant physiology: structure of cell walls, membrane function, and metabolic activities (*19*). This nutrient is one of those responsible for the changes in concentration and metabolism of phenolic compounds in vascular plants, due to its effects on polyphenol oxidase activity (*20*). This enzyme is normally bound to membranes or walls in a latent form and becomes active when released under boron-deficient conditions, which leads to phenolic compounds accumulation (*19*).

Sulfur is often referred to as the fourth major plant nutrient, as it is an essential component of important metabolic and structural compounds (*21*). Whereas nitrogen is mainly used for structural macromolecules, sulfur plays critical roles in the catalytic or electrochemical functions of the biomolecules in cells. Sulfur is found in amino acids, oligopeptides, vitamins and cofactors, and a variety of secondary compounds, such as glucosinolates in the Brassicaceae (*2, 22*).

Although vegetables or vegetable food commodities are produced under conventional practices, there has been increasing interest in organic products. Consumer perception is that these products are healthier than the conventional ones, with higher nutritional value and reduced pesticide residues, with the advantage of being produced in a sustainable agricultural practice less harmful to the environment (*7, 12, 23*). When several quality aspects of organically and conventionally grown plant-derived foods were compared, only small and inconsistent differences were found (*1*). In previous works comparing organic or conventional production, *B. oleracea* var. *costata* grown under organic practices showed a slight tendency to increased phenolics levels (*5, 6*), although it was not possible to conclude that this corresponds to higher antioxidant potential (*24*). However, this tendency was not evident for other *Brassica* species (*23*).

Due to the important role of phenolic compounds as health protective agents, the aim of this work was to evaluate the influence of fertilizers, namely, organic (Dix10) and chemicals (nitrogen, boron, and sulfur), on *B. oleracea* var. *costata* phenolic composition and biomass production. In addition, the interference of the level of chemical fertilizer was also checked.

MATERIALS AND METHODS

Standards and Reagents. *p*-Coumaric and sinapic acids were from Sigma (St. Louis, MO) and kaempferol 3-*O*-glucoside and kaempferol 3-*O*-rutinoside from Extrasynthése (Genay, France). Analytical grade formic and acetic acids were purchased from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

Plant Material and Treatments. Tronchuda cabbage (*B. oleracea* var. *costata*) plants were grown under different fertilization regimes. The experimental work was carried out in one field located in Bragança, northeastern Portugal (41° 48′ N, 6° 44′ W). The field had an inclination inferior to 5% and was turned up to the northeast. Sowing occurred in the middle of June 2005, in a greenhouse (22 ± 2 °C, 80% humidity). Young plants were transplanted to the field at the end of August, spaced at 0.8 × 0.5 m between and within rows. Before the fertilization treatments, the soil was loamy textured with 0.83% organic matter, a pH (H_2O) of 5.2, and median phosphorus (54 mg of P_2O_5/kg) and high potassium (126 mg of K_2O/kg) levels. Eight treatments were established: a control (C), without any fertilization, and one with Dix10 (Crimolara, Portugal), an authorized organic amendment (10% total N, 3% K_2O, 3% P_2O_5, 2.5% CaO, 0.6% MgO, and 30.5 mg of B/kg); N1 and N2, with 80 and 160 kg of N/ha (80 kg/ha soil application + 80 kg/ha side dress application in mid-October), respectively; B1 and B2, with 2.2 and 4.4 kg of B/ha, respectively; and S1 and S2, with 37.3 and 74.6 kg of S/ha, respectively. All conventional fertilizer regimens received 80 kgof N/ha to ensure plant growth, with the exception of the N2

treatment. The fertilizers were simultaneously applied at the beginning of the growth season. The conventional fertilizers used were urea, borax, and magnesium sulfate (ADP, Portugal). Phosphorus (150 kg of super 18/ha) and potassium (50 kg of KCl/ha) were also used in these fields. Twelve cabbages were planted for each experimental treatment.

Samples were collected on three occasions during the growth season: in mid-November 2005 (a), mid-December 2005 (b), and mid-January 2006 (c). At each harvesting date and for each fertilization regimen three plants were randomly collected from three different plots. All samples were collected in the morning, at the same hour. After harvesting, the plants were immediately transported to the laboratory and weighed, and external and internal leaves were separated. Care was taken to choose plants and leaves of similar developmental stage: internal leaves, looking pale yellow and tender, were separated from the external ones, which presented a dark green color and were no longer actively expanding, although not yet senescent.

Each analyzed sample corresponds to the mixture of the three plants developed and collected in the same conditions. Two hours maximum after their collection, the samples were frozen at −20 °C and then lyophilized (Labconco 4.5 Freezone apparatus, Kansas City, MO). The freeze-dried samples were powdered and kept in a desiccator in the dark, until they were subjected to phenolics extraction. To evaluate the dry weight, three plants of each treatment of the third harvesting time were dried at 65 °C until constant weight (6 days).

Phenolic Compounds Extraction. An aqueous extract was used for the phytochemical characterization: ca. 3.0 g of powdered *B. oleracea* var. *costata* leaves was boiled for 15 min in 300 mL of water and then filtered over a Büchner funnel. The resulting extract was lyophilized, and a yield of ca. 1.4 g was obtained. The lyophilized extract was kept in a desiccator, in the dark. The lyophilized extract was redissolved in water, in triplicate, immediately before the HPLC-DAD analysis.

HPLC-DAD Quantitative Analysis of Phenolics. As internal and external leaves exhibit distinct phenolics composition (*5, 25*), to achieve a better separation of the compounds the HPLC gradients used for the analysis of the two kinds of leaves were different. The analysis of phenolic compounds was carried out as previously reported (*5, 25*) using a HPLC unit (Gilson) and a 250 × 4.6 mm i.d., 5 μm Spherisorb ODS2 column (Waters, Milford, MA), protected with a 4 × 4 mm Spherisorb ODS2 guard column. For the internal leaves analysis, elution was performed using acetic acid 1% (A) and methanol (B) as solvents, starting with 20% B and using a gradient to obtain 50% B at 30 min, 80% B at 37 min, and 100% B at 42 min. For the external leaves the solvent system was a mixture of formic acid 5% (A) and methanol (B), with the following gradient: 0 min, 10% B; 25 min, 20% B; 40 min, 50% B; 45 min, 50% B; 46 min, 90% B; 50 min, 90% B; 55 min, 100% B; 58 min, 100% B; and 60 min, 10% B. The flow rate was 1 mL/min, and the injection volume was 20 μL. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200–400 nm, and chromatograms were recorded at 330 nm. The data were processed on Unipoint system software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic Compounds identification was based on the retention time and UV spectra of each peak, in comparison with data published before for external (*5*) and internal (*25*) *B. oleracea* var. *costata* leaves. Quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. With the exception of kaempferol 3-*O*-glucoside, which was quantified as itself, the kaempferol derivatives were quantified as kaempferol 3-*O*-rutinoside, 3- and 4-*p*-coumaroylquinic acids were quantified as *p*-coumaric acid, and sinapic acid derivatives were quantified as sinapic acid, because none of them was commercially available.

Statistical Analysis. Principal component analysis (PCA) was applied to the results. PCA was performed by the SPSS program (version 15.0). PCA was applied separately for external and internal leaves. Individual concentrations of each phenolic compound and fresh weight of the plant were selected as variables.

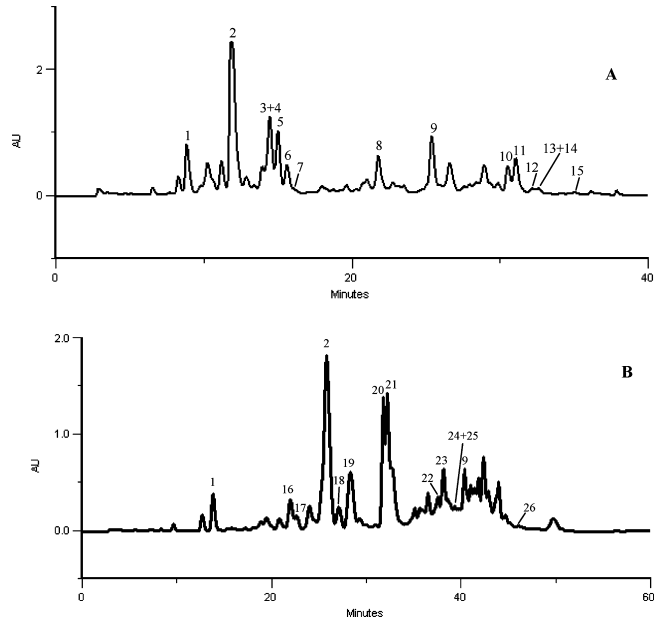

Figure 2. HPLC-DAD chromatogram of phenolic compounds of an aqueous extract of *B. oleracea* var. *costata* (control sample from the first harvesting): (**A**) internal leaves; (**B**) external leaves. Detection at 330 nm. Compounds: (**1**) 3-*p*-coumaroylquinic acid; (**2**) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (**3**) sinapoyl glucoside acid; (**4**) kaempferol 3-*O*-(sinapoyl)-sophoroside-7-*O*-glucoside; (**5**) kaempferol 3-*O*-(feruloyl)-sophoroside-7-*O*-glucoside; (**6**) kaempferol 3-*O*-(*p*-coumaroyl)-*O*-sophoroside-7-*O*-glucoside; (**7**) 4-*p*-coumaroylquinic acid; (**8**) sinapic acid; (**9**) kaempferol 3-*O*-sophoroside; (**10**) 1,2-disinapoylgentiobioside; (**11**) 1-sinapoyl-2-feruloylgentiobiose; (**12**) isomer of 1,2-disinapoylgentiobioside; (**13**) 1,2,2′-trisinapoylgentiobiose; (**14**) 1,2′-disinapoyl-2-feruloylgentiobiose; (**15**) isomer of 1,2-disinapoylgentiobioside; (**16**) kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside; (**17**) kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside; (**18**) kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside; (**19**) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside; (**20**) kaempferol 3-*O*-(sinapoyl/caffeoyl)sophoroside-7-*O*-glucoside; (**21**) kaempferol 3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside; (**22**) kaempferol 3-*O*-sophorotrioside; (**23**) kaempferol 3-*O*-(sinapoyl)sophoroside; (**24**) kaempferol 3-*O*-(feruloyl)sophorotrioside; (**25**) kaempferol 3-*O*-(feruloyl)sophoroside; (**26**) kaempferol 3-*O*-glucoside.

RESULTS AND DISCUSSION

Changes in Biomass. Tronchuda cabbages grown with no fertilization presented the lowest fresh weight, at all harvesting dates (**Figure 1**): on average, samples developed under organic amendment or chemical fertilization exhibited 1.4- and 2.8-fold the weight of those grown with no fertilization, respectively. Additionally, samples developed under organic amendment exhibited lower weight than the ones grown with traditional fertilization. Phenological development is driven by temperature and by photoperiod: a short photoperiod is associated with reduced peak radiation, reduced daily radiation, lower mean temperature, and lower minimum temperature. Of these, low minimum temperature would advance phenology in thermal time, whereas both short photoperiod and low radiation could have the reverse effect of slowing phenological development in *Brassica* species (*26*). Although a period of 3 months was considered, the harvesting time did not have a relevant effect on fresh weight in any of the samples.

All plants presented similar moisture contents, ranging from ca. 84 to 88%, with no important differences among samples grown under different agronomic practices.

Table 1. Quantification of *Brassica oleracea* var. *costata* Internal Leaves Phenolic Compounds (Milligrams per Kilogram, Dry Basis)[a]

		phenolic compound													
treatment	harvest[b]	**1**	**2**	**3 + 4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13 + 14**	**15**	**Σ**
C	a	1263.6	8977.2	3785.8	2718.5	1413.2	288.6	911.8	4035.6	541.1	813.5	164.4	249.3	126.6	25289
		16.72	*101.70*	*18.05*	*44.57*	*6.77*	*21.97*	*7.94*	*100.02*	*1.70*	*1.92*	*4.03*	*4.80*	*0.55*	
Dix10	a	961.6	5709.4	4008.9	1453.4	992.6	74.6	547.1	1839.1	355.0	383.8	37.5	41.1	38.0	16442
		20.80	*131.67*	*105.12*	*20.79*	*48.33*	*6.13*	*0.32*	*38.76*	*0.57*	*4.29*	*1.99*	*0.87*	*3.53*	
N1	a	473.7	1615.8	1154.4	486.3	199.4	39.4	391.1	474.4	394.4	286.8	102.7	133.3	29.1	5781
		4.60	*17.04*	*5.26*	*3.16*	*2.33*	*0.72*	*1.24*	*9.02*	*4.59*	*8.36*	*1.55*	*2.45*	*1.63*	
N2	a	766.9	2700.3	1378.4	971.7	290.4	108.2	450.7	721.5	275.2	264.2	42.4	73.9	34.2	8078
		0.68	*40.14*	*53.00*	*34.38*	*27.65*	*8.36*	*38.42*	*53.73*	*4.42*	*1.18*	*4.57*	*3.52*	*1.05*	
B1	a	966.3	1262.5	954.7	414.0	178.4	107.6	432.6	666.2	437.5	362.5	99.7	103.3	7.5	5993
		60.91	*94.14*	*7.56*	*6.71*	*4.93*	*0.75*	*51.85*	*9.94*	*11.75*	*14.92*	*1.97*	*14.52*	*2.24*	
B2	a	610.6	2444.5	1156.6	810.7	240.9	79.4	346.9	1034.9	296.7	274.5	50.3	78.0	21.5	7446
		17.34	*21.70*	*71.89*	*6.78*	*4.81*	*1.36*	*14.15*	*15.45*	*5.55*	*0.59*	*1.37*	*1.33*	*0.97*	
S1	a	547.6	nq	353.9	83.0	60.3	49.6	191.8	649.5	25.3	18.3	11.2	22.4	26.6	2040
		14.29		*10.87*	*3.66*	*1.65*	*1.59*	*5.21*	*22.23*	*1.22*	*1.28*	*0.16*	*1.68*	*2.04*	
S2	a	604.7	1499.1	1082.7	572.1	308.8	76.7	286.3	684.2	413.6	376.1	94.3	157.2	66.5	6222
		5.08	*4.95*	*9.06*	*23.49*	*26.19*	*6.99*	*27.38*	*28.36*	*2.65*	*0.46*	*6.68*	*16.16*	*12.16*	
C	b	390.8	5821.4	3230.5	1554.9	502.9	150.2	330.7	2618.8	262.3	369.9	51.3	79.0	12.5	15375
		17.34	*153.72*	*85.16*	*136.73*	*30.12*	*22.80*	*6.48*	*360.83*	*8.10*	*2.81*	*3.90*	*0.36*	*0.12*	
Dix10	b	527.0	1935.9	1884.8	631.1	144.5	nq	238.8	1332.3	184.7	172.1	50.6	106.8	15.2	7224
		19.20	*29.62*	*42.75*	*13.53*	*6.30*		*6.48*	*18.46*	*3.87*	*0.32*	*0.64*	*6.86*	*0.97*	
N1	b	262.8	279.1	246.3	33.2	37.0	34.6	195.6	220.7	147.7	82.7	22.5	39.3	6.5	1608
		10.17	*8.49*	*0.67*	*4.05*	*0.18*	*1.38*	*10.59*	*8.40*	*9.39*	*0.15*	*2.35*	*2.50*	*0.41*	
N2	b	296.6	591.9	972.2	247.3	87.9	36.6	337.8	509.5	254.7	137.6	38.6	53.9	6.5	3571
		13.92	*9.01*	*8.95*	*1.18*	*1.04*	*1.31*	*6.59*	*75.12*	*6.83*	*1.65*	*0.92*	*0.12*	*0.60*	
B1	b	342.9	2021.5	1335.1	454.9	66.9	25.8	313.5	1158.6	333.1	244.9	71.1	84.2	7.1	6460
		21.53	300.94	16.95	17.27	4.23	0.47	1.21	54.30	6.47	2.88	1.20	0.22	0.07	
B2	b	554.6	957.5	756.1	317.0	125.2	88.3	398.0	235.0	365.8	248.1	53.6	64.3	11.1	4175
		1.11	*6.45*	*10.21*	*2.85*	*4.49*	*3.15*	*13.83*	*10.13*	*14.82*	*0.86*	*3.91*	*0.39*	*0.47*	
S1	b	396.3	820.8	915.2	323.3	87.8	27.3	323.8	372.1	309.0	337.7	43.3	135.1	7.2	4099
		28.35	*9.43*	*17.94*	*16.85*	*6.10*	*1.15*	*19.06*	*48.07*	*11.20*	*14.29*	*0.59*	*9.66*	*0.44*	
S2	b	435.7	505.6	805.3	230.4	43.9	35.9	219.2	716.0	272.9	205.0	51.6	83.9	23.0	3629
		12.47	*17.21*	*3.42*	*0.63*	*0.07*	*0.45*	*2.74*	*23.40*	*16.47*	*4.56*	*2.02*	*3.85*	*1.64*	
C	c	451.7	9749.2	5589.6	2121.7	865.6	nq	489.2	5191.2	589.8	553.8	112.1	169.4	108.6	25992
		23.94	*247.88*	*266.18*	*129.77*	*46.86*		*42.96*	*386.98*	*44.84*	*63.18*	*8.20*	*22.36*	*15.30*	
Dix10	c	347.8	1018.6	1037.0	291.4	99.0	12.0	147.1	999.2	161.7	152.3	33.5	52.4	21.7	4374
		0.17	*38.08*	*12.04*	*6.00*	*2.01*	*0.43*	*10.18*	*21.61*	*15.63*	*4.09*	*5.21*	*0.97*	*0.45*	
N1	c	393.3	2371.7	1395.8	467.9	206.8	6.9	357.7	2029.1	263.6	87.3	20.1	79.7	43.6	7723
		12.73	*71.71*	*40.39*	*11.75*	*6.17*	*0.56*	*6.84*	*25.64*	*6.46*	*99.26*	*9.16*	*27.62*	*2.88*	
N2	c	311.7	1130.9	1055.2	328.4	107.4	17.0	245.4	1340.1	222.6	91.2	20.5	27.2	23.9	4922
		6.52	*23.03*	*11.17*	*11.72*	*3.87*	*0.44*	*11.41*	*62.93*	*5.26*	*4.65*	*2.45*	*1.24*	*2.41*	
B1	c	281.8	488.5	545.9	173.1	64.9	37.9	234.2	784.3	170.5	89.8	27.7	48.0	27.9	2974
		8.89	*34.21*	*41.61*	*27.87*	*3.66*	*1.52*	*3.13*	*0.24*	*2.51*	*3.77*	*0.16*	*1.00*	*0.80*	
B2	c	282.3	787.4	1111.3	329.6	409.0	61.0	271.8	1069.1	149.0	103.7	32.3	88.7	61.2	4756
		4.89	*45.40*	*86.11*	*13.90*	*474.37*	*4.91*	*8.82*	*52.55*	*5.46*	*10.80*	*0.13*	*1.66*	*0.89*	
S1	c	374.3	1365.4	1069.4	401.0	164.1	11.3	271.4	1608.0	226.0	197.7	45.5	86.1	60.5	5881
		1.21	*17.22*	*26.01*	*0.34*	*6.06*	*0.13*	*1.12*	*1.48*	*6.48*	*2.47*	*0.10*	*15.03*	*0.51*	
S2	c	224.4	117.8	340.8	73.7	42.9	13.7	288.3	213.7	131.1	72.9	12.7	18.3	6.4	1557
		20.92	*10.73*	*27.89*	*7.69*	*0.41*	*1.48*	*25.80*	*18.44*	*8.13*	*10.50*	*2.06*	*2.04*	*0.22*	
	mean	502.9	2355.3	1506.9	645.4	280.8	62.8	342.5	1271.0	282.6	246.9	53.7	86.5	33.0	7567
	max	1263.6	9749.2	5589.6	2718.5	1413.2	288.6	911.8	5191.2	589.8	813.5	164.4	249.3	126.6	25992
	min	224.4	nq	246.3	33.2	37.0	nq	147.1	213.7	25.3	18.3	11.2	18.3	6.4	1557
	SD	257.3	2654.6	1313.7	672.8	344.5	62.9	156.1	1203.1	132.0	177.6	36.8	53.1	31.7	6622

[a] Results are expressed as mean of three determinations; *italic figures* indicate standard deviation. Σ, sum of the determined phenolic compounds. [b] Harvesting times: a, mid-November 2005; b, mid-December 2005; c, mid-January 2006.

Phenolic Compounds. The quality and quantity of phenolics are influenced by the maturity of the leaves, being different in young and old ones (*27*). In the case of tronchuda cabbage analyzed samples, the phenolic composition of internal (younger) and external leaves (older) was considerably different in terms of qualitative profile and total amounts, as was observed before (*5, 25*). In the internal leaves were found 15 phenolics, of which 5 were kaempferol derivatives and 10 were cinnamic acid derivatives (**Figure 2A**). The external leaves were characterized by the presence of 3-*p*-coumaroylquinic acid and 13 kaempferol derivatives (**Figure 2B**). Only 3-*p*-coumaroylquinic acid (**1**), kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**2**), and kaempferol 3-*O*-sophoroside (**9**) were found in both leaves. Kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**2**) was the major compound in both kinds of leaves, kaempferol 3-*O*-sophoroside (**9**) being also an important one.

The external leaves total phenolic compounds amount was, in general, much higher than that of the internal ones (**Tables 1** and **2**). This can be ascribed to the fact that the production of phenolics is usually lower during rapid growth of the younger leaves, increasing significantly thereafter, when the photosyn-

Table 2. Quantification of *Brassica oleracea* var. *costata* External Leaves Phenolic Compounds (Millirams per Kilogram, Dry Basis)[a]

		phenolic compound													
treatment	harvest[b]	**1**	**16**	**17**	**2**	**18**	**19**	**20**	**21**	**22**	**23**	**24 + 25**	**9**	**26**	Σ
C	a	659.7	1180.2	482.0	9702.6	823.5	2694.1	3422.0	3905.0	546.6	1303.8	324.9	2319.4	8.8	27372
		7.37	*37.01*	*4.82*	*184.03*	*20.24*	*117.82*	*36.19*	*126.30*	*143.63*	*128.77*	*46.53*	*42.74*	*0.61*	
Dix10	a	469.3	1109.9	359.8	7042.8	625.2	3142.0	2825.5	2531.0	1017.7	1418.1	1753.0	2505.5	39.1	24839
		5.62	55.25	28.99	20.08	26.92	23.86	1036.92	70.74	58.09	21.80	84.85	68.99	1.29	
N1	a	260.1	1160.5	254.3	5460.0	357.9	2645.0	2533.2	3233.1	313.1	762.2	766.6	1462.1	nq	19208
		2.55	*16.10*	*46.22*	*28.39*	*7.83*	*15.57*	*20.24*	*35.84*	*87.94*	*180.63*	*273.99*	*393.58*		
N2	a	387.0	532.2	213.5	5016.3	414.9	1526.2	1798.2	2028.2	971.4	1430.1	439.6	1735.4	6.4	16499
		1.46	*4.73*	*0.31*	*25.01*	*7.24*	*17.75*	*11.29*	*22.62*	*17.20*	*20.11*	*26.79*	*39.59*	*0.04*	
B1	a	592.0	292.1	647.6	6545.0	906.0	922.3	2438.4	2740.4	1485.1	3016.3	831.8	3262.2	nq	23679
		2.04	*34.97*	*73.43*	*31.67*	*143.61*	*143.61*	*16.45*	*1260.77*	*82.92*	*101.46*	*47.14*	*194.46*		
B2	a	397.6	979.6	181.2	5472.1	305.9	1906.9	1479.8	2241.9	823.2	1606.1	1331.2	1784.4	nq	18510
		21.07	*63.84*	*9.73*	*444.22*	*18.04*	*120.35*	*81.98*	*93.63*	*39.68*	*88.94*	*142.57*	*38.60*		
S1	a	541.9	416.3	1188.3	5793.7	443.1	2333.7	2094.0	3216.6	924.6	1127.9	413.3	2226.0	118.6	20838
		15.80	*15.93*	*50.01*	*42.60*	*0.98*	*92.45*	*104.88*	*356.42*	*113.76*	*135.80*	*27.12*	*252.26*	*9.70*	
S2	a	325.6	926.4	228.7	6742.0	406.6	1730.3	2012.1	2449.0	928.7	1028.5	767.9	2657.0	69.6	20272
		13.90	*20.34*	*3.20*	*84.71*	*13.56*	*31.92*	*22.92*	*50.93*	*20.19*	*31.11*	*32.50*	*68.87*	*3.19*	
C	b	228.6	2495.9	545.9	8163.1	1907.9	3008.3	3332.2	3472.2	856.5	1868.9	890.9	2355.7	13.2	29139
		0.24	*121.59*	*1.59*	*522.85*	*91.27*	*51.65*	*285.09*	*52.04*	*23.82*	*181.45*	*89.67*	*169.29*	*0.19*	
Dix10	b	349.8	1511.2	771.7	6352.3	2280.5	1862.0	3880.2	5624.0	449.3	3112.0	892.6	1953.3	nq	29039
		0.05	*118.97*	*60.16*	*313.61*	*88.33*	*59.63*	*285.73*	*444.68*	*2.15*	*143.93*	*70.33*	*26.26*		
N1	b	245.6	875.5	428.6	3623.5	518.1	1566.5	1748.2	2703.7	597.0	2570.6	782.5	1619.0	3.8	17283
		0.09	*70.51*	*39.58*	*6.40*	*18.58*	*108.06*	*115.21*	*24.66*	*98.58*	*142.40*	*4.94*	*263.01*	*1.36*	
N2	b	183.4	1353.2	823.9	4713.8	833.3	1249.9	2933.3	3112.3	663.7	1283.7	821.4	1550.5	11.5	19534
		0.94	*52.54*	*36.30*	*143.47*	*29.93*	*44.90*	*78.59*	*87.99*	*20.32*	*52.90*	*32.61*	*33.46*	*0.51*	
B1	b	198.9	938.0	456.5	5253.7	788.8	1084.6	2495.9	2946.0	1091.9	2244.4	858.7	2123.4	13.0	20494
		8.48	*61.11*	*18.64*	*49.13*	*40.49*	*77.27*	*54.86*	*156.18*	*22.37*	*17.68*	*46.09*	*10.27*	*1.62*	
B2	b	349.1	1071.7	855.0	4740.7	835.6	1904.4	2655.8	2905.6	585.9	950.4	852.4	1004.5	17.7	18729
		12.51	*3.91*	*105.59*	*199.88*	*160.52*	*198.50*	*101.81*	*6.91*	*12.04*	*48.51*	*45.78*	*39.04*	*1.11*	
S1	b	158.4	1281.1	246.6	2650.2	611.8	1681.2	1550.6	3939.8	861.2	1952.8	853.1	1131.4	38.8	16957
		7.63	*382.39*	*33.25*	*103.31*	*29.42*	*103.32*	*65.50*	*108.82*	*8.46*	*118.26*	*15.21*	*31.59*	*5.11*	
S2	b	285.6	745.9	232.2	4634.3	673.3	1009.9	2411.8	3195.8	795.9	1346.1	1968.7	2102.0	93.0	19495
		25.76	*29.57*	*6.27*	*73.57*	*46.57*	*69.85*	*172.86*	*192.96*	*4.09*	*52.97*	*125.56*	*153.42*	*1.63*	
C	c	116.8	2968.3	553.2	7478.5	2390.3	2574.8	3533.0	3463.3	454.1	2663.5	850.4	1457.4	135.3	28639
		3.19	*17.06*	*0.73*	*36.22*	*11.92*	*30.88*	*9.68*	*11.11*	*1.88*	*1.39*	*4.81*	*8.46*	*1.10*	
Dix10	c	306.8	2126.8	633.7	6864.2	2235.8	1980.0	4787.2	3862.1	1015.0	2304.8	1206.6	2980.4	131.2	30434
		1.15	*7.49*	*48.28*	*11.71*	*11.98*	*26.12*	*145.88*	*37.17*	*51.88*	*280.52*	*86.00*	*129.55*	*27.42*	
N1	c	156.7	2239.7	237.3	7594.9	1365.5	1410.5	3233.1	2482.0	304.9	1763.5	541.8	2803.2	76.6	24210
		2.66	*22.80*	*8.98*	*109.46*	*54.32*	*61.20*	*69.65*	*37.86*	*17.36*	*222.09*	*95.29*	*96.74*	*4.04*	
N2	c	154.8	1216.1	287.6	5643.6	999.0	1019.0	2701.3	2371.0	265.0	445.2	151.2	787.3	nq	16041
		1.75	*10.75*	*4.66*	*201.11*	*11.42*	*18.75*	*40.75*	*19.36*	*2.87*	*89.31*	*35.38*	*61.41*		
B1	c	262.4	792.5	217.6	4721.2	625.8	876.8	3230.5	2269.8	744.3	2205.7	626.9	1265.0	73.0	17911
		6.61	*78.10*	*23.11*	*162.08*	*62.28*	*91.68*	*517.40*	*454.78*	*85.83*	*316.60*	*65.45*	*110.88*	*3.51*	
B2	c	153.0	741.7	242.5	6826.5	645.7	945.7	3712.9	3333.9	800.2	2306.3	729.2	1621.2	18.1	22077
		3.24	*10.39*	*9.35*	*158.18*	*33.12*	*11.26*	*72.75*	*130.01*	*35.86*	*154.22*	*84.94*	*220.28*	*7.97*	
S1	c	182.5	1957.3	359.3	7275.1	1391.6	1343.4	3786.0	3594.9	784.4	2395.5	599.5	1592.2	50.8	25312
		0.47	*30.12*	*7.50*	*45.07*	*35.96*	*28.22*	*145.54*	*11.38*	*172.19*	*381.32*	*123.82*	*140.05*	*0.30*	
S2	c	61.0	804.4	213.6	5083.0	747.5	701.7	2827.6	2703.0	1403.6	3327.8	1328.9	3266.5	nq	22469
		0.11	*12.45*	*9.40*	*176.26*	*75.68*	*55.77*	*20.14*	*68.06*	*42.75*	*38.91*	*36.89*	*63.59*		
	mean	292.8	1238.2	444.2	5974.7	963.9	1713.3	2809.3	3096.9	778.5	1851.4	857.6	1981.9	51.0	22041
	max	659.7	2968.3	1188.3	9702.6	2390.3	3142.0	4787.2	5624.0	1485.1	3327.8	1968.7	3266.5	135.3	30434
	min	61.0	292.1	181.2	2650.2	305.9	701.7	1479.8	2028.2	265.0	445.2	151.2	787.3	nq	16041
	SD	154.1	671.1	263.0	1545.4	631.7	711.8	821.6	770.5	313.1	774.3	418.2	688.5	44.9	4430

[a] Results are expressed as mean of three determinations; *italic figures* indicate standard deviation. Σ, sum of the determined phenolic compounds. [b] Harvesting times: a, mid-November 2005; b, mid-December 2005; c, mid-January 2006.

thetic capacity of the newly matured leaves is highest and they are no longer growing (*18*). The kaempferol derivatives present in internal leaves varied between ca. 51 and 90% of total phenolics. The sinapic acid derivatives represented ca. 7.2−34%. In this group, sinapic acid (**8**) was, for most of the samples, the major compound, representing ca. 2.0−18.5%. The *p*-coumaroylquinic acid derivatives (compounds **1** and **7**) varied between 1.7 and 18.5%. Sinapic acid derivatives **12**, **13** + **14**, **15**, and 4-*p*-coumaroylquinic acid (**7**) were minor compounds in all samples.

As referred to above, external leaves were characterized by the presence of 13 kaempferol derivatives and 3-*p*-coumaroylquinic acid (compound **1**), which represented ca. 0.3−2.6% of total phenolics. The general pattern of kaempferol glycosylation was substitution in the 3-position with sophoroside, with some compounds simultaneously acylated with cinnamic acids, and in the 7-position with glucose. With the exception of sample S1, from December 2005 (collection date b), kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**2**) was the major compound in all of the samples, accounting for more than ca. 20% of total phenolics. Kaempferol 3-*O*-glucoside (**26**) was found only in very low amounts (less than ca. 0.6%).

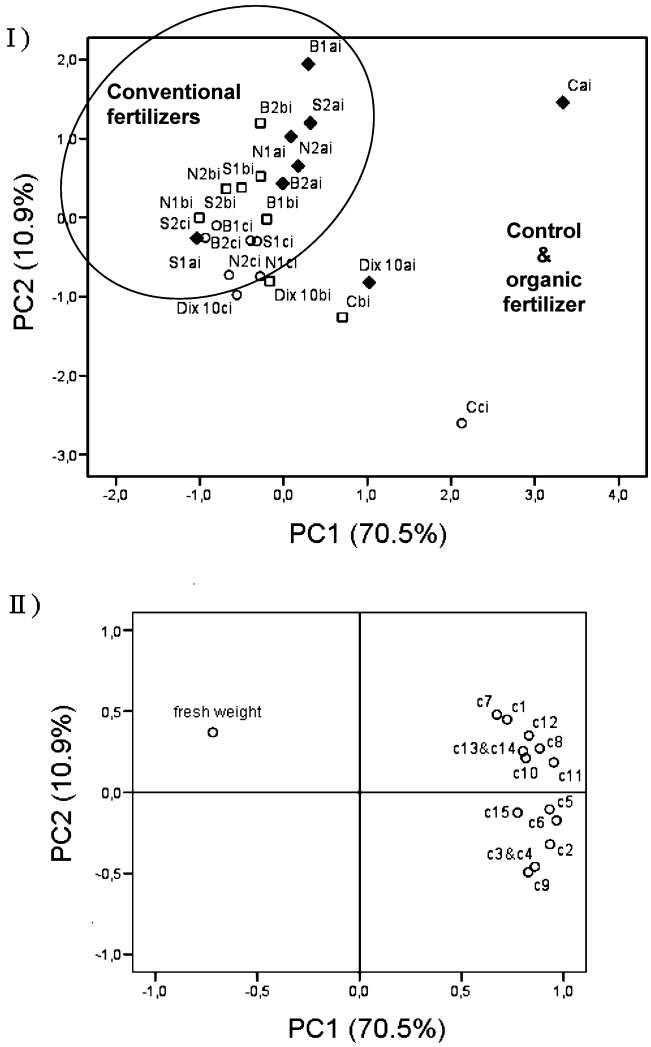

Figure 3. Principal component analysis of phenolic compounds of *B. oleracea* var. *costata* internal leaves: (**I**) scores plot; (**II**) loadings plot. Samples: C, control; Dix 10, organic amendement; N1, 80 kg of N/ha; N2, 160 kg of N/ha; B1, 2.2 kg of B/ha; B2, 4.4 kg of B/ha; S1, 37.3 kg of S/ha; S2, 74.6 kg of S/ha. Harvesting dates: a, mid-November 2005; b, mid-December 2005; c, mid-December 2006.

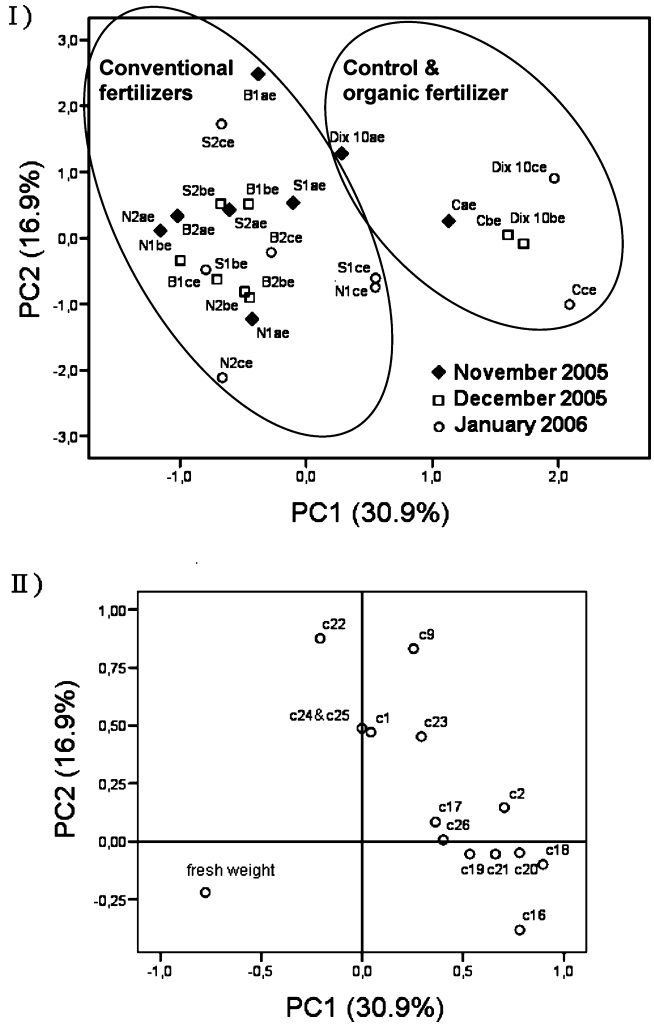

Figure 4. PC1 versus PC2 plot of phenolic compounds of *B. oleracea* var. *costata* external leaves: (**I**) scores plot; (**II**) loadings plot. Identification of the samples is as in **Figure 3**. Harvesting dates: a, mid-November 2005; b, mid-December 2005; c, mid-December 2006.

General Effects of Fertilization. The patterns of phenolics did not change qualitatively in leaves of *B. oleracea* var. *costata* grown under different agronomical practices. However, some differences were noticed in quantitative terms (**Tables 1** and **2**). The highest levels of these compounds were detected in control samples, both in internal and in external leaves: external and internal leaves developed without any fertilization contained 1.4- and 4.6-fold more phenolic compounds than the ones that received conventional fertilizer, respectively, and the internal leaves presented 2.4 times more phenolics than the ones grown with organic amendment. Although total phenolics contents were higher in samples with no or organic fertilization, the amounts found per plant, considering the fresh weight, were not very different in all samples. This can be explained by dilution by leaf growth, instead of increased phenolics production (*18*). Generally, external leaves had a lower dispersion, and this is more evident in terms of the total amount of phenolic compounds, which varied between 16.0 and 30.4 g/kg for external leaves and between 1.6 and 26 g/kg in the internal ones.

Principal component analysis (PCA) was performed to assess the relationships between phenolic compounds, agronomical practices, and harvesting time. PCA was applied separately to both types of leaves due to their qualitative different phenolic profiles, with only three compounds in common, as referred to above (compounds **1**, **2**, and **9**).

For internal leaves, PCA yielded two PCs (with eigenvalues higher than 1) that accounted for 81.3% of the total variance in the data (**Figure 3**). PC1 and PC2 explained 70.5 and 10.9% of the total variance, respectively. For external leaves (**Figures 4** and **5**), five PCs with eigenvalues greater than 1 accounted for 79.9% of the total variance in the data. To simplify the analysis of the results, and considering the scree plot, only the first three PCs were retained, explaining 62.9% of the total variance (30.9, 16.9, and 14.2%, respectively).

For both types of leaves (**Figures 3** and **4**), cabbages produced with organic fertilizer and control were quite effectively separated from those of chemical fertilizations, although they were not clustered according to the amounts and different fertilizers provided in each agronomical practice. Scores of leaves collected in November 2005 (a) fall more separated from those of samples collected in December 2005 (b) or January 2006 (c) (**Figures 3** and **5**).

Effects of Organic Fertilization. In general, samples from organic fertilization exhibited a tendency for higher total phenolics content than those from conventional practices, collected at the same time (**Tables 1** and **2**). The higher concentrations of phenolic compounds in those samples can be explained by the increased

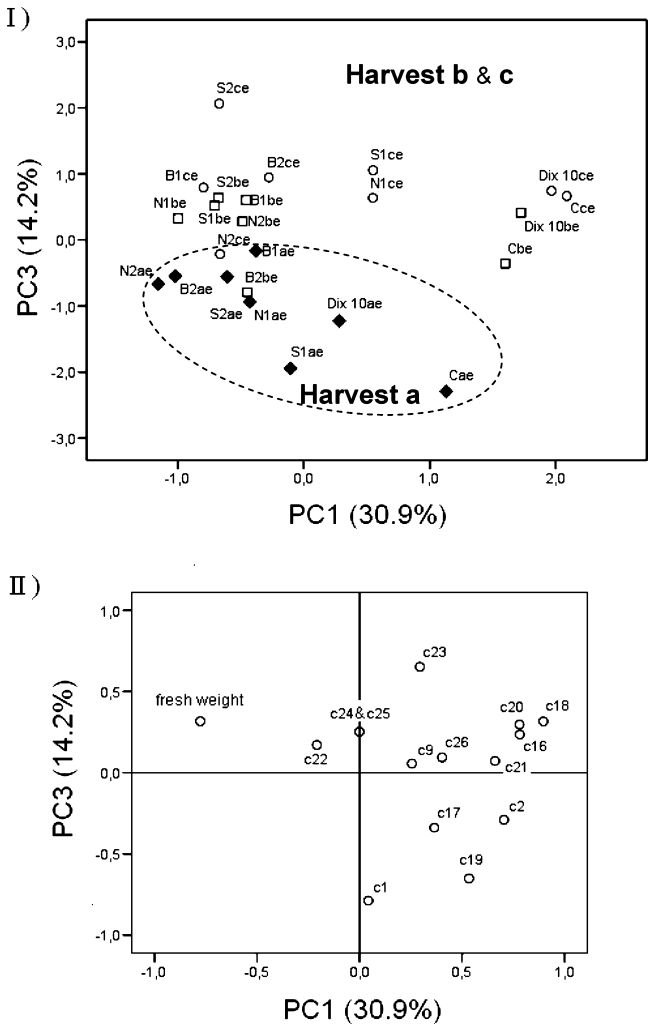

Figure 5. PC1 versus PC3 plot of phenolic compounds of *B. oleracea* var. *costata* external leaves: (**I**) scores plot; (**II**) loadings plot. Identification of the samples is as in **Figure 3**.

environmental stress caused by the lack of nutrients (*7, 8, 11, 15, 23*), once in this case the degree of mineralization of nitrogen could be insufficient to provide the nutrients needed for the rapid growth of young leaves.

In the internal leaves from organic fertilization, the kaempferol derivatives varied between 79 and 90% of total phenolics. This proportion decreased in samples from conventional fertilization, in some cases to 50%, being more pronounced in samples fertilized simultaneously with nitrogen and sulfur. It seems that in the internal leaves the organic fertilization induces the acetate/shikimate pathway, resulting in higher production of flavonoids, whereas the conventional fertilization preferentially leads to the shikimate pathway alone, resulting in more phenolic acids. In external leaves the kaempferol derivatives were always higher than 97%.

Effects of Nitrogen Fertilization. Fertilization with nitrogen usually accelerates the vegetative growth of plants, although increased nitrogen availability may decrease the levels of phenolic compounds (*14*). A trend for increased biomass production (**Figure 1**) and decreased phenolic compounds concentrations (**Tables 1** and **2**) was observed with the moderate level of nitrogen (N1 samples). When the results were compared with those obtained with samples treated with higher levels of nitrogen (N2), no relevant differences were found (**Figures 3−5**).

Low nitrogen availability is the most common growth-limiting factor for primary production of plants (*1, 13, 14*). In most crops, yield is clearly improved by nitrogen fertilization, particularly when it is limiting the genetic growth potential (*13, 28*). Maybe with the moderate level of nitrogen the plants reached the genetically determined capacity to form biomass (*28*). Therefore, these results indicate that it may be possible to obtain good yields in *B. oleracea* var. *costata* growth and high phenolics content with minimized nitrogen fertilization. The risk of nitrogen leakage from the soil can thereby be reduced, which has a positive impact on the environment (*27*).

Effects of Sulfur Fertilization. Application of sulfur together with nitrogen had no significant effects on the growth or phenolics metabolism of *B. oleracea* var. *costata* (**Figures 1** and **3−5** and **Tables 1** and **2**). Sulfur assimilation by plants responds dynamically to changes in environmental conditions and to sulfur supply (*22*). As a consequence, phenolic compounds can vary with sulfur fertilization, due to its effects on phenolic enzymes polyphenol oxidase and peroxidase (*29*). Yield and quality responses to applied sulfur are likely especially on light sandy soils as atmospheric sulfur inputs are being reduced (*21*). Maybe in the fields where this experiment was developed, the sulfur inputs, other than the agronomical application, were still enough to ensure good plant development.

Effects of Boron Fertilization. Boron deficiency is the most widespread of all the micronutrient deficiencies (*20*), although it can be toxic when present in excess (*30*). The primary effect of boron deficiency is the reduction of cell enlargement in growing tissues (*30*). Other effects are the cessation of root elongation, reduced leaf expansion, and loss of fertility (*31*). Boron fertilization of *B. oleracea* var. *costata*, a hardy crop, did not considerably affect its growth and phenolic composition (**Tables 1** and **2** and **Figures 3−5**).

Effects of Harvesting Time. Plants harvested in November, generally with a higher content of phenolic compounds (**Tables 1** and **2**), showed a good separation from the samples harvested later (**Figures 3** and **5**). These results may be explained by UV irradiation: the leaves of these samples developed earlier in the winter, with a larger photoperiod, and it is known that this factor induced flavonoids (particularly kaempferol derivatives) and sinapate esters in *Arabidopsis* (*32*) and caused an overall increase in the amount of soluble flavonoids in *Brassica napus* (*33*). The same was observed for broccoli, for which, in general, rich sulfur fertilization and longer exposure to sunlight lead to higher concentrations of phenolic compounds (*29*).

In the case of internal leaves phenolics content, the difference between organic and N1 fertilization found in the last collection was smaller than those observed for the samples collected earlier. Maybe at the date of the last collection the plants are already adapted to the fertilization conditions, leading to smaller difference between these two regimes. Additionally, the internal leaves of sample S1, from the first harvesting date (a), showed a distinct phenolic profile, especially with regard to the major compounds kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**2**), which was not quantifiable, and kaempferol 3-*O*-sophoroside (**9**), the percentage of which almost corresponds to the sum of compounds **2** and **9** in the other samples. Therefore, this sample did not cluster with the other samples from the same date (**Figure 3**).

In terms of individual phenolics, the concentrations of 3-*p*-coumaroylquinic (**1**) and 4-*p*-coumaroylquinic (**7**) acids were significantly higher in the first harvesting and for all treatments of internal leaves (**Figure 3**, **II**).

On the other hand, no important differences were observed in the percentage of kaempferol 3-*O*-sophoroside (**9**), a major compound in both kinds of leaves. The kaempferol glycosylation

with sophorotrioside, which was found only in external leaves, notably increased from the first sampling time to the last sampling.

The differences found for total phenolics composition at the three harvesting dates were not relevant, being not possible to indicate a time for which the harvest of tronchuda cabbage samples with high phenolics contents is favored. These results show that for these compounds the optimum harvest time spans a large period during the winter.

In conclusion, we can say that *B. oleracea* var. *costata* can be grown with different fertilization regimens (organic or chemical) without affecting the phenolic profile, although the total amount of phenolic compounds is higher under organic practices. Despite being possible to grow the plant without any kind of fertilization, its development will be greatly affected, but no decrease in phenolics biosynthesis will be observed.

By comparison with the application of a moderate dose of nitrogen (80 kg/ha), the use of a high nitrogen quantity (160 kg/ha) or the simultaneous fertilization with a moderate nitrogen dose and boron or sulfur does not considerably affect phenolics and biomass of the plants. Therefore, it is possible to grow tronchuda cabbage without excess fertilizers, with highest amounts of phenolics and reduced environment pollution. The effect of fertilization in other essential nutrients cannot be forgotten and deserves to be explored.

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4.7. *Brassica oleracea* var. *costata*: Comparative study on organic acids and biomass production with other cabbage varieties

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Brassica oleracea var. *costata*: comparative study on organic acids and biomass production with other cabbage varieties

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Abstract

BACKGROUND: A study was undertaken to evaluate the effect of agronomic practices, harvesting time and leaf age on the organic acid composition and biomass production of *Brassica oleracea* L. var. *costata* DC (tronchuda cabbage). Samples were cultivated under eight different fertilisation regimes (two levels each of nitrogen, boron and sulfur, an organic fertiliser and no fertiliser) and collected at three different times.

RESULTS: Principal component analysis of the data indicated significant differences. Three principal components with an eigenvalue higher than one accounted for 79.0% of the total variance of the data set. Samples obtained with conventional fertilisation were characterised by the highest values of fresh weight. External leaves showed higher total organic acid and malic acid contents than internal leaves, while the latter were characterised by higher proportions of citric acid. For consecutive harvests, total organic acid concentration decreased in both external and internal leaves.

CONCLUSION: The use of a conventional fertilisation regime (nitrogen, boron or sulfur) improved the growth of *B. oleracea* var. *costata* without affecting its organic acid profile. However, for consecutive harvests, total organic acid concentration was observed to decrease independently of the agronomic practices tested. Leaf age influenced the quantitative composition of organic acids.
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Keywords: *Brassica oleracea* L. var. *costata* DC; organic acids; fertilisation; principal component analysis

INTRODUCTION

Plants have the ability to accumulate organic acids in the cell vacuoles[1] in quantities that depend on their type of carbon fixation, nutritional status and degradative activities. The predominant organic acids are also determined by factors such as the age of the plant and the type of tissue.[2,3]

Among the organic acids found in plants, those from the tricarboxylic acid (Krebs) cycle, namely citric, aconitic, isocitric, ketoglutaric, succinic, fumaric, malic and oxalacetic acids, can be distinguished. All these acids occur in catalytic amounts in plant tissues, although only citric and malic acids are regularly accumulated.[1] Shikimic and quinic acids, not present in the Krebs cycle, are also of interest, being precursors of aromatic compounds in plants. Several organic acids have been found in previous studies involving *Brassica* species.[4–7]

Apart from the above, malic, citric, aconitic and fumaric acids carry out other functions in plant metabolism. Malic and citric acids are the most abundant organic acids in *Brassica oleracea* L. var. *costata* DC, commonly known as tronchuda cabbage,[4–6] and their storage in the tissues and possible release are regulated by the plant cells independently.[8] Malic acid is a versatile compound that can easily be transported across the cellular membranes or stored in the vacuoles. It can perform various functions, such as acting as a substrate for mitochondrial adenosine 5′-triphosphate (ATP) production, providing nicotinamide adenine dinucleotide (NADH) to the cytosol, maintaining the cytosolic pH value[8,9] and acting as an osmoticum and as a counterion for potassium or sodium.[2,8] Citric acid plays an important role in the translocation of iron in the roots and in its long-distance transport through the xylem to the leaves.[10] Aconitic acid is involved in carbohydrate biosynthesis in the glioxalate cycle and presents allelopathic activity.[11] Fumaric acid can be metabolised to yield energy and carbon skeletons for production of other compounds and may also help in the maintenance of cellular pH and turgor pressure.[12]

The synthesis and intracellular accumulation of oxalic acid in plants are implicated in cell calcium homeostasis,[13] although from a nutritional standpoint this acid is considered to be an antinutrient because it renders calcium, and sometimes other minerals, unavailable for nutritional absorption.[14] However,

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B. oleracea varieties have been shown to be a good source of calcium owing to their combination of high levels of calcium with low oxalic acid content.[15]

Shikimic acid, identified in the common aromatic amino acid pathway leading to L-phenylalanine, L-tyrosine and L-tryptophan, is used by higher plants as a protein building block and as a precursor for a large number of secondary metabolites.[16]

Plant growth and productivity are determined by photosynthesis and respiration[17] and can be improved by various agronomic practices. Conventional practices of fertilisation involve the use of nitrogen, sulfur and boron. Fertilisation with nitrogen stimulates the synthesis of organic acids, which in turn are used for the synthesis of amino acids and several secondary metabolites, e.g. glucosinolates and phenylpropanoid compounds, or stored as counteranions.[18–20] Sulfur is an essential component of important metabolic and structural compounds.[21,22] While nitrogen is used mainly for structural macromolecules, sulfur plays critical roles in catalytic and electrochemical functions of biomolecules in cells. Sulfur is found in amino acids, oligopeptides, vitamins and cofactors and in a variety of secondary products such as glucosinolates in the Brassicaceae.[23–25] Boron is required for normal growth and development of all higher plants and is thought to be involved in cell wall structures, membrane functions and metabolic activities.[26–28] In contrast to conventional systems in which synthetic fertilisers containing directly available inorganic nitrogen are used, organic systems rely on the activity of a diverse soil ecosystem to make nitrogen available to plants.[29,30]

The aim of this work was to study the effect of different fertilisation practices, harvesting time and leaf age on the organic acid composition and biomass production of *B. oleracea* var. *costata*. The data obtained were subjected to principal component analysis.

EXPERIMENTAL

Standards and reagents

cis-Aconitic, citric, fumaric, L-(-)-malic, oxalic and shikimic acids were obtained from Sigma (St Louis, MO, USA). Sulfuric acid (H_2SO_4) was purchased from Merck (Darmstadt, Germany). The water used was pretreated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Plant material and treatments

Tronchuda cabbage (*B. oleracea* L. var. *costata* DC) plants were grown under different fertilisation regimes. The experiment was conducted in a single field located in Bragança in northeastern Portugal (41° 48′N, 6° 44′W). Sowing was carried out in the middle of June 2005 in a greenhouse (22 ± 2 °C, 80% relative humidity). Young plants were transplanted to the field at the end of August, spaced at 0.8 m × 0.5 m between and within rows. Before the fertilisation treatments the soil was loamy textured with 8.3 g kg^{-1} organic matter, pH (H_2O) 5.2 and medium phosphorus (54 mg P_2O_5 kg^{-1}) and high potassium (126 mg K_2O kg^{-1}) levels. Eight treatments were established: a control (C), without any fertilisation, and one with Dix10 (Crimolara, Lisboa, Portugal), an authorised organic amendment (100 g total N, 30 g K_2O, 30 g P_2O_5, 25 g CaO, 6 g MgO and 30.5 mg B kg^{-1}); N1 and N2, with 80 and 160 kg N ha^{-1} (80 kg ha^{-1} soil application + 80 kg ha^{-1} side-dress application in mid-October) respectively; B1 and B2, with 2.2 and 4.4 kg B ha^{-1} respectively; and S1 and S2, with 37.3 and 74.6 kg S ha^{-1} respectively. The Dix10 and conventional fertiliser regimes provided equivalent amounts of nitrogen, with the exception of the N2 treatment. The fertilisers were applied at the beginning of the growth season. The conventional fertilisers used were urea, borax and magnesium sulfate (ADP, Lisboa, Portugal). Phosphorus (150 kg Super 18 ha^{-1}) and potassium (50 kg KCl ha^{-1}) were also used in these treatments.

Samples were collected on three different occasions during the growth season, in mid-November 2005 (a), mid-December 2005 (b) and mid-January 2006 (c). At each harvesting date and for each fertilisation regimen, three plants were selected randomly from three different plots. After harvesting, the plants were immediately transported to the laboratory and weighed, then external (older) and internal (younger) leaves were separated. Care was taken to choose plants and leaves of similar developmental stage: internal leaves looking pale yellow and tender were separated from external leaves which presented a dark green colour and were no longer actively expanding although not yet senescent.

Each analysed sample corresponded to a mixture of three plants developed and collected under the same conditions. At 2 h maximum after their collection, the samples were frozen at −20 °C and then lyophilised (Labconco 4.5 Freezone apparatus, Kansas City, MO, USA). The freeze-dried samples were powdered and kept in a desiccator in the dark until they were subjected to organic acid extraction. In order to evaluate the dry weight, three plants of each treatment from the third harvesting time were dried at 65 °C until constant weight (∼6 days).

Organic acid extraction

An aqueous extract was used for phytochemical characterisation. Approximately 3 g of powdered tronchuda cabbage leaves were boiled for 15 min in 300 mL of water and then filtered over a Büchner funnel. The resulting extract was lyophilised and a yield of *ca* 1.4 g was obtained. The lyophilised extract was kept in a desiccator in the dark.

Analysis of organic acids

Each extract was analysed by high-performance liquid chromatography (HPLC) with UV detection as reported previously,[5] with some modifications. The system consisted of an analytical HPLC unit (Gilson, Villiers Le Bel, France) with a Nucleogel Ion 300 OA (Macherey-Nagel, Düren, Germany) ion exclusion column (300 mm × 7.7 mm) in conjunction with a column-heating device set at 30 °C. Elution was carried out isocratically at a solvent flow rate of 0.2 mL min^{-1} using 0.005 mol L^{-1} H_2SO_4. The injection volume was 20 μL.

Detection was performed with a UV detector set at 214 nm. Organic acid quantification was achieved via the absorbances recorded in the chromatograms relative to external standards. The peaks in the chromatograms were integrated using a default baseline construction technique.

Principal component analysis

Multivariate analysis of variance (MANOVA) was performed to evaluate the effects of the studied factors – leaf age, fertilisation and harvesting time – on organic acid composition and biomass production. Principal component analysis (PCA) was performed to better understand the relations between organic acid quantitative composition and the above factors. Both analyses were carried out using SPSS 15, (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

The various samples of tronchuda cabbage showed the same organic acid profile, which was characterised by the presence of

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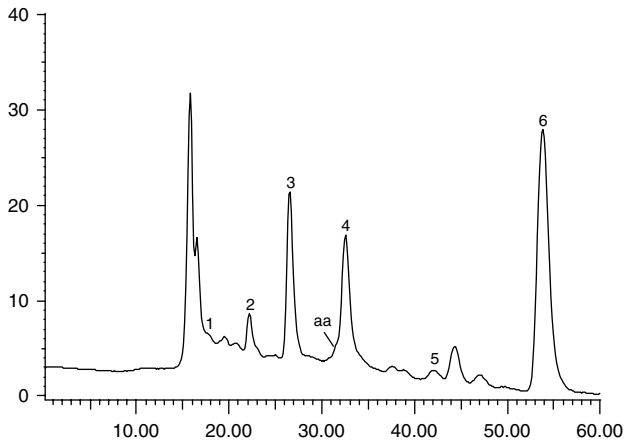

Figure 1. HPLC-UV chromatogram (detection at 214 nm) of aqueous extract of organic acids from internal leaves of tronchuda cabbage: 1, oxalic acid; 2, aconitic acid; 3, citric acid; 4, malic acid; 5, shikimic acid; 6, fumaric acid; aa, ascorbic acid.

oxalic, aconitic, citric, malic, shikimic and fumaric acids (Fig. 1), as reported previously in *Brassica* species[4–7] In the analysed samples, ascorbic acid was found only in traces, which could be explained by the drastic extraction procedure used. This method was chosen since it mimics the usual conditions of cabbage consumption. In addition, the analytical procedures were not specific for ascorbic acid, as we intended to determine the highest possible number of compounds. It is possible that ascorbic acid became oxidised, making it impossible to quantify.

The qualitative profile was not affected by the maturity of the leaves, harvesting time or fertilisation employed. Compared with *Brassica rapa* var. *rapa*, tronchuda cabbage shows some differences, such as the absence of ketoglutaric acid and the presence of oxalic acid.[31]

The total organic acid content of the leaves (internal and external) ranged between 729 and 9986 mg kg^{-1} fresh weight (Tables 1 and 2). The major compounds determined were malic acid, which represented 23.6–90.6% of the total organic acids, and citric acid, which varied between 8.9 and 73.9%. The other compounds, fumaric (<3.6%), aconitic (<3.1%), oxalic (<2.6%) and shikimic (<0.2%) acids, appeared in much lower proportions. Despite the variations seen within *B. rapa* var. *rapa*[31] and *B. oleracea* var. *costata*, malic and citric acids are the main organic acids in both species, which is not surprising considering that these compounds are commonly accumulated in plants.[1]

Relative amounts of each organic acid (%), total organic acid concentration and fresh weight of the plant were selected as variables (Tables 1 and 2) for MANOVA and PCA.

MANOVA showed significant differences between the organic acid profiles of the samples grown under different fertilisation practices in terms of fresh weight of the plant ($P < 0.001$). Significant differences were also found among the samples collected at the three harvesting dates in respect of the relative concentrations of aconitic ($P < 0.001$), fumaric ($P < 0.001$), oxalic ($P < 0.001$) and shikimic ($P < 0.05$) acids, total concentration of organic acids ($P < 0.001$) and fresh weight of the plant

Table 1. Quantification of organic acids in internal leaves of tronchuda cabbage (mg kg^{-1} fresh weight)[a]

Treatment	Harvest[b]	Oxalic	Aconitic	Citric	Malic	Shikimic	Fumaric	$\sum$
C	a	NQ	7.5 ± 0.05	539.0 ± 4.91	171.8 ± 13.09	NQ	10.6 ± 0.31	729
	b	4.8 ± 0.53	0.6 ± 0.01	414.4 ± 2.24	512.1 ± 3.51	0.5 ± 0.03	7.5 ± 0.01	939
	c	NQ	19.5 ± 0.11	463.1 ± 33.52	252.3 ± 21.46	0.8 ± 0.24	21.2 ± 0.37	757
Dix10	a	5.0 ± 0.01	20.8 ± 0.01	674.2 ± 10.82	259.6 ± 3.18	0.6 ± 0.02	35.4 ± 0.13	996
	b	16.5 ± 0.20	1.4 ± 0.01	539.3 ± 8.83	3799.2 ± 58.44	2.3 ± 0.19	10.0 ± 0.16	4369
	c	NQ	28.2 ± 0.04	578.2 ± 0.01	283.3 ± 21.74	1.4 ± 0.32	19.2 ± 0.42	910
N1	a	31.8 ± 0.09	4.9 ± 0.16	1630.0 ± 14.69	910.0 ± 12.78	2.1 ± 0.01	35.7 ± 0.45	2615
	b	23.3 ± 4.32	29.5 ± 0.02	1539.5 ± 2.95	1062.1 ± 1.64	4.0 ± 0.07	26.9 ± 0.06	2685
	c	NQ	35.1 ± 0.04	807.4 ± 3.09	673.8 ± 39.81	1.5 ± 0.12	50.7 ± 0.26	1568
N2	a	9.2 ± 0.31	18.7 ± 0.15	1412.7 ± 22.42	1292.8 ± 49.56	5.1 ± 0.57	23.8 ± 0.23	2766
	b	NQ	2.6 ± 0.14	1278.8 ± 8.24	6276.7 ± 44.11	2.2 ± 0.13	26.4 ± 0.02	7587
	c	NQ	31.3 ± 0.29	1117.4 ± 3.88	705.5 ± 13.92	2.0 ± 0.28	44.7 ± 0.93	1901
B1	a	22.6 ± 0.74	10.1 ± 0.64	1360.6 ± 29.57	1239.8 ± 21.96	2.4 ± 0.02	37.6 ± 0.29	3068
	b	10.2 ± 0.10	0.7 ± 0.03	693.7 ± 5.31	2467.0 ± 9.84	4.8 ± 0.05	17.7 ± 0.01	3194
	c	NQ	13.0 ± 0.52	1095.2 ± 34.90	1029.4 ± 45.30	3.3 ± 0.15	41.2 ± 0.18	2182
B2	a	11.3 ± 0.30	14.8 ± 0.20	1427.4 ± 15.81	1671.3 ± 69.26	3.8 ± 0.22	43.1 ± 0.82	3172
	b	NQ	20.2 ± 0.02	985.6 ± 60.17	967.4 ± 74.38	3.0 ± 0.29	35.8 ± 0.67	2407
	c	NQ	33.5 ± 0.14	1068.1 ± 0.60	696.9 ± 1.68	1.2 ± 0.13	59.9 ± 0.01	1860
S1	a	NQ	2.3 ± 0.22	765.2 ± 9.73	1926.1 ± 25.63	3.2 ± 0.16	23.3 ± 0.23	2720
	b	26.7 ± 1.36	3.9 ± 0.08	890.8 ± 6.56	9048.1 ± 52.29	2.0 ± 0.40	15.0 ± 0.01	9986
	c	NQ	40.3 ± 0.27	1262.2 ± 19.33	774.9 ± 109.04	1.2 ± 0.07	50.8 ± 0.66	2129
S2	a	84.9 ± 1.16	11.6 ± 0.42	1447.2 ± 30.94	1667.3 ± 225.21	2.5 ± 0.17	14.5 ± 0.38	3263
	b	21.2 ± 2.42	11.3 ± 0.16	1119.5 ± 7.80	1319.3 ± 2.89	0.8 ± 0.04	36.4 ± 0.12	2508
	c	NQ	18.2 ± 0.08	1071.3 ± 4.30	904.7 ± 0.10	1.4 ± 0.09	37.6 ± 0.04	2033

[a] Values are mean ± standard deviation of three determinations; NQ, not quantified; $\sum$, sum of determined organic acids.
[b] Harvesting times: a, mid-November 2005; b, mid-December 2005; c, mid-January 2006.

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Table 2. Quantification of organic acids in external leaves of tronchuda cabbage (mg kg^{-1} fresh weight)[a]

Treatment	Harvest[b]	Oxalic	Aconitic	Citric	Malic	Shikimic	Fumaric	$\sum$
C	a	13.8 ± 1.11	0.2 ± 0.01	1586.8 ± 60.76	2411.0 ± 7.18	3.6 ± 0.07	50.2 ± 0.52	4065
	b	2.0 ± 0.12	5.1 ± 0.13	1109.3 ± 2.88	1294.7 ± 25.77	1.0 ± 0.01	31.3 ± 0.35	2443
	c	8.5 ± 0.44	10.6 ± 0.29	485.1 ± 55.39	900.5 ± 28.66	1.7 ± 0.08	40.5 ± 0.19	1447
Dix10	a	14.4 ± 1.55	19.0 ± 0.36	1598.7 ± 52.05	2623.5 ± 28.6	1.9 ± 0.20	55.4 ± 1.17	4312
	b	24.8 ± 0.28	2.0 ± 0.05	919.6 ± 2.13	5656.5 ± 2.40	0.2 ± 0.03	15.6 ± 0.13	6619
	c	NQ	19.9 ± 0.01	760.8 ± 6.62	884.0 ± 1.57	1.5 ± 0.09	41.2 ± 0.01	1708
N1	a	19.5 ± 5.10	9.3 ± 0.35	1159.7 ± 35.03	5124.4 ± 92.09	4.4 ± 0.90	58.2 ± 0.15	6375
	b	6.5 ± 0.24	6.7 ± 0.08	758.4 ± 7.75	2545.9 ± 35.00	1.7 ± 0.20	41.0 ± 0.04	3360
	c	12.8 ± 0.17	9.0 ± 0.08	641.4 ± 6.51	2184.0 ± 18.20	2.7 ± 0.07	46.2 ± 0.09	2896
N2	a	20.5 ± 1.65	8.1 ± 0.14	2387.7 ± 13.97	4987.8 ± 45.41	1.7 ± 0.04	44.2 ± 0.40	7450
	b	4.4 ± 0.05	2.5 ± 0.03	670.7 ± 3.35	3405.2 ± 8.92	1.2 ± 0.09	26.4 ± 0.14	4110
	c	NQ	4.0 ± 0.02	565.1 ± 12.73	3075.4 ± 9.07	2.5 ± 0.05	33.4 ± 0.14	3680
B1	a	106.2 ± 5.92	5.8 ± 0.49	2342.6 ± 34.68	5505.8 ± 250.68	4.0 ± 0.39	35.0 ± 0.86	7999
	b	2.1 ± 0.12	0.5 ± 0.01	269.2 ± 7.07	1386.6 ± 6.84	0.8 ± 0.09	18.0 ± 0.57	1677
	c	12.6 ± 0.92	8.5 ± 0.24	485.7 ± 10.05	1211.1 ± 19.93	3.4 ± 0.36	50.5 ± 0.33	1772
B2	a	18.7 ± 0.72	12.7 ± 0.47	1369.3 ± 1.89	3871.2 ± 19.83	2.1 ± 0.06	47.7 ± 0.02	5322
	b	8.2 ± 0.49	4.3 ± 0.19	956.5 ± 17.19	3033.2 ± 86.82	2.4 ± 0.05	27.7 ± 0.38	4032
	c	NQ	9.0 ± 0.01	613.8 ± 2.07	1247.6 ± 15.80	2.0 ± 0.07	52.1 ± 0.02	1924
S1	a	17.3 ± 3.85	9.9 ± 0.49	1402.1 ± 22.05	4105.8 ± 53.66	1.7 ± 0.35	17.0 ± 0.27	5554
	b	9.8 ± 0.23	16.8 ± 0.30	901.5 ± 35.95	1830.0 ± 3.70	2.1 ± 0.20	60.6 ± 0.21	2821
	c	7.2 ± 0.29	9.6 ± 0.02	504.1 ± 5.89	1005.6 ± 6.44	2.0 ± 0.12	42.4 ± 0.01	1571
S2	a	NQ	6.9 ± 0.02	2084.1 ± 13.57	5252.6 ± 37.26	5.2 ± 0.52	36.0 ± 0.45	7385
	b	NQ	4.7 ± 0.03	628.5 ± 0.34	3030.8 ± 19.15	1.8 ± 0.04	54.0 ± 0.14	3720
	c	NQ	12.4 ± 0.03	519.8 ± 19.87	1194.4 ± 15.89	3.0 ± 0.54	50.6 ± 0.74	1780

[a] Values are mean ± standard deviation of three determinations; NQ, not quantified; $\sum$, sum of determined organic acids.
[b] Harvesting times: a, mid-November 2005; b, mid-December 2005; c, mid-January 2006.

($P < 0.05$). Lastly, leaf age also influenced the organic acid composition significantly in terms of the relative contents of aconitic ($P < 0.001$), citric ($P < 0.001$) and malic ($P < 0.001$) acids and total concentration of organic acids ($P < 0.05$).

These differences were confirmed by PCA. Three principal components (PCs) with an eigenvalue higher than one accounted for 79.0% of the total variance of the data set. PC1 explained 49.4% of the total variance, PC2 explained 15.3% and PC3 explained 14.3%. The score plots of PC1 *vs* PC2 and PC1 *vs* PC3 are presented in Figs 2 and 3 respectively.

Figures 2 and 3 show that, in general, PC1 separated the external and internal leaves quite effectively. Variables related to PC2 influenced more the separation of the cabbages produced with organic fertiliser (Dix10) and no fertiliser (C) from those obtained using chemical fertilisers (B1, B2, N1, N2, S1 and S2), although they did not cluster them according to the amounts of different fertilisers provided in each agronomic practice (Fig. 2). Lastly, samples harvested in January 2006 (c) were also separated by PC1 from those previously collected in November 2005 (a) or December 2005 (b), all of them grown using chemical fertilisation.

Figures 4 and 5 show the loading plots obtained for PC1 *vs* PC2 and PC1 *vs* PC3 respectively. The study of the loadings, coefficients that represent the weight of the original variable in the PCs, enabled us to better understand the relations between the samples and the variables studied.

PC1 correlated positively with aconitic (0.86), citric (0.89) and fumaric (0.81) acids and negatively with malic acid (−0.91) and total organic acids (−0.82), while the other variables did not have a

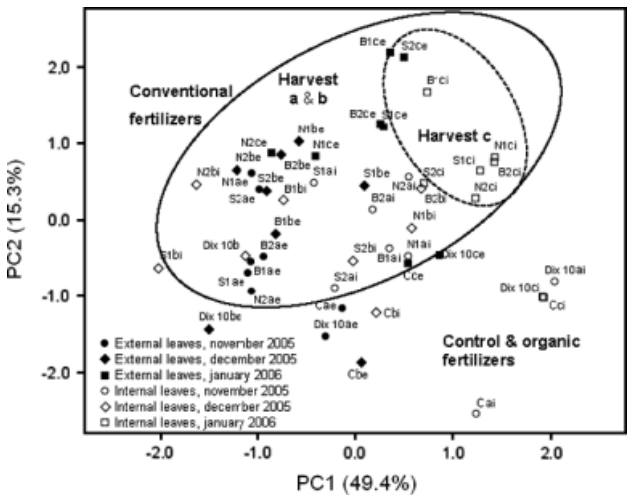

Figure 2. PCA of organic acids of tronchuda cabbage: PC1 *vs* PC2 score plot. Samples: C, control; Dix 10, organic amendement; N1, 80 kg N ha^{-1}; N2, 160 kg N ha^{-1}; B1, 2.2 kg B ha^{-1}; B2, 4.4 kg B ha^{-1}; S1, 37.3 kg S ha^{-1}; S2, 74.6 kg S ha^{-1}. Harvesting times: a, mid-November 2005; b, mid-December 2005; c, mid-December 2006. Leaves: i, internal; e, external.

high influence (<0.46). Fresh weight of the plant (0.77) and oxalic acid (0.92) were the variables with the highest contribution to PC2 and PC3 respectively, while the other variables had loading values lower than 0.59.

Considering this statistical analysis, the effects of fertilisation, leaf age and harvesting time on the quantitative composition of

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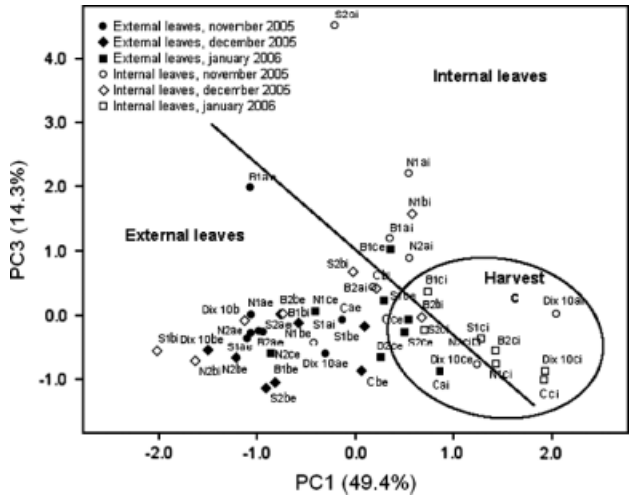

Figure 3. PCA of organic acids of tronchuda cabbage: PC1 *vs* PC3 score plot. See Fig. 2 for identification of samples.

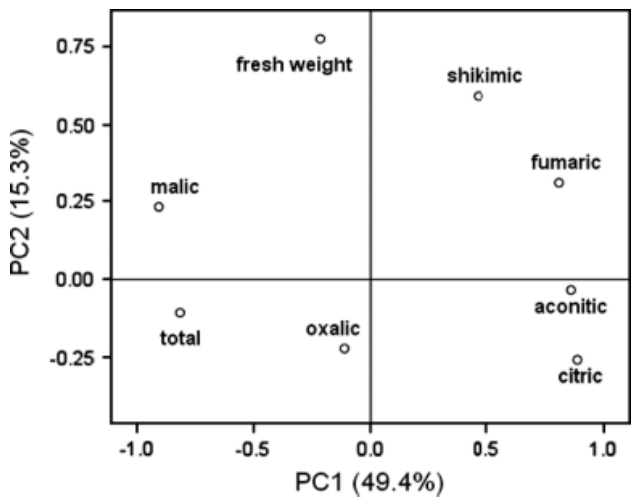

Figure 4. PCA of organic acids of tronchuda cabbage: PC1 *vs* PC2 loading plot.

the cabbage samples were studied in more detail and are reported below.

Effects of fertilisation

Figure 2 shows that the samples fertilised with boron (B1 and B2), nitrogen (N1 and N2) and sulfur (S1 and S2) were quite well differentiated from those obtained with organic fertilisation (Dix10) and no fertilisation (C). The samples developed with conventional fertilisers were mainly located on the positive side of PC2 and the negative side of PC1. According to the loading plot (Fig. 4) and Table 3, the major contribution for the differentiation of these samples was given by the fresh weight of the plant: samples obtained with conventional fertilisation were characterised by the highest values of fresh weight. Fresh weight of the samples obtained with conventional fertilisation varied between 1214.5 and 1901.3 g, while for the other samples it ranged between 476.5 and 756.8 g.

During fast vegetative growth the reduction of nitrate and the synthesis of carboxylate and amino acids are high. Nitrate reduction implies the formation of toxic alkaline ions, which cannot

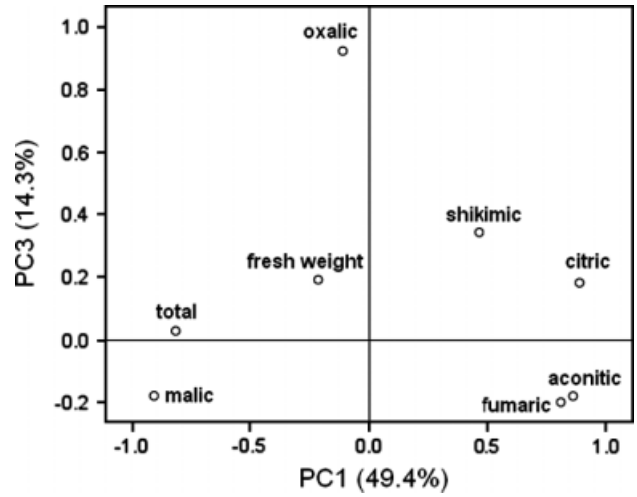

Figure 5. PCA of organic acids of tronchuda cabbage: PC1 *vs* PC3 loading plot.

Table 3. Fresh weight of tronchuda cabbage[a]

Treatment	Fresh weight (g) Mid-November 2005	Mid-December 2005	Mid-January 2006
C	476.5 ± 32.79	551.8 ± 210.17	562.3 ± 35.23
Dix10	756.8 ± 165.30	733.2 ± 219.45	726.9 ± 139.16
N1	1262.8 ± 326.60	1526.1 ± 722.08	1267.7 ± 405.95
N2	1253.3 ± 147.41	1901.3 ± 559.64	1427.8 ± 230.82
B1	1227.0 ± 97.48	1214.5 ± 185.01	1713.1 ± 676.97
B2	1378.5 ± 529.18	1604.0 ± 429.56	1579.7 ± 420.16
S1	1314.5 ± 348.38	1240.8 ± 357.97	1672.7 ± 331.95
S2	1449.1 ± 101.38	1549.9 ± 339.67	1544.0 ± 123.07

[a] Values are mean ± standard deviation of three replicates.

be efficiently eliminated by the cells. To maintain its homeostasis, the plant synthesises organic acids, mainly malic and citric acids.[3] The use of boron, nitrogen and sulfur seemed to improve the growth of tronchuda cabbage without decreasing its total organic acid concentration. For each type of leaf the sum of individual organic acid concentrations was higher in conventionally fertilised samples than in control samples at each collection time. However, in comparison with organic fertilisation, conventional fertilisation led to higher organic acid content only in samples collected in November and January.

These differences were clearer when the amounts found per plant, taking into account the fresh weight of each plant, were studied. Considering the same type of leaf, the amount of organic acids in conventionally fertilised samples was between 1.5 and 28 times higher than that in control samples at each collection time, and between 2.2 and 6.2 times higher than that in organically fertilised samples collected in November and January.

Effects of leaf age

The organic acid profile can change with the age of the plant.[2,3] In Fig. 2 it can be seen that external leaves appeared mainly on the left side of the plot (negative PC2). External and internal leaves were more clearly separated in the score plot of PC1 *vs* PC3 (Fig. 3).

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The corresponding loading plot (Fig. 5) showed that the most influential variables to differentiate the two types of leaves were total organic acids and malic and citric acids. Considering plants grown under the same fertilisation conditions and collected at the same time, it was observed that, in general, external leaves presented higher total organic acid and malic acid contents, while internal leaves were characterised by lower total organic acid and higher citric acid contents. Previous works have already revealed some relation between malic acid content and maturity degree. In fact, it was noticed that this acid increased continuously with maturation,[32] which is in accordance with the higher content found in external (older) leaves.

External leaves showed levels of total organic acids ranging between 1447 and 7999 mg kg^{-1} (Table 2), while internal leaves showed levels of 729–9986 mg kg^{-1} (Table 1). The greatest difference in the total concentration of organic acids between external and internal leaves was found in the samples harvested in November (a), especially in the control samples (C) and those obtained with organic fertilisation (Dix10): external leaves showed a total organic acid concentration 5.6 times higher than internal leaves for the unfertilised samples and 4.3 times higher for the organic samples.

As mentioned above, external leaves were richer in malic acid and internal leaves exhibited higher levels of citric acid, while the other compounds were minor in all samples. This effect was especially observed in the samples harvested in November and January. Therefore, for external leaves (Table 2), malic acid was always the major compound (51.8–85.5%) and citric acid the second most abundant (13.9–45.4%), while, for internal leaves (Table 1), malic acid (23.6–90.6%) or citric acid (8.9–73.9%) was the major compound, depending on the sample. Thus the type and age of the plant tissue proved to have an influence on the predominant organic acid, as reported previously.[2]

Effects of harvesting time

Figure 2 shows the differences between the samples fertilised with boron, nitrogen and sulfur according to the harvesting time. The samples harvested in January (c) appeared in the first quadrant (positive PC1 and positive PC2), quite well separated from the other samples harvested in November (a) and December (b), which appeared more mixed in the third and fourth quadrants (negative PC1). The corresponding loading plot (Fig. 4) revealed that the most influential variables to differentiate the two groups were shikimic, fumaric and aconitic acids, which characterised the samples harvested in January, and malic acid and total organic acids, which characterised the samples harvested in November and December. The separation of the samples collected in January was also evident in Fig. 3. In January a lower organic acid content is generally seen, which may be ascribed to reduced metabolic activity due to the reduced temperatures and photoperiod.

In general, it was observed that the total organic acid content of the analysed cabbage decreased for consecutive harvests (Tables 1 and 2) using conventional fertilisation, although some differences were observed according to the type of fertiliser employed. For external leaves (Table 2), this was strictly followed: samples obtained with the same agronomic practices showed the highest contents of total organic acids when they were harvested in November (5322–7999 mg kg^{-1}) and the lowest levels when they were harvested in January (1571–3680 mg kg^{-1}). The concentration decreased by 24–79% from the first to the second harvest and by 51–78% from the first to the third harvest.

For internal leaves (Table 1), samples B2 and S2 showed the highest levels of total organic acids when they were harvested in November (3172 and 3263 mg kg^{-1} respectively), while samples N1, N2, B1 and S1 showed the highest values when they were harvested in December (2685–9986 mg kg^{-1}). The organic acid concentration of samples B2 and S2 decreased by 22–24% from the first to the second harvest, whereas that of the other samples increased by 2.7–267%.

The samples harvested in January always showed the lowest values (1568–2182 mg kg^{-1}), corresponding to a decrease of 18–41% relative to the samples harvested in November.

The samples harvested in January showed greater differences than those harvested in November and December, with higher levels of the minor compounds aconitic (0.1–3.1%) and fumaric (0.9–3.2%) acids in both types of leaves and shikimic acid (0.1–0.2%) in external leaves. Besides, most of them showed lower levels of malic acid (31.1–83.6%). Fumaric acid concentration was previously found to increase.[12]

On other hand, data obtained for the samples receiving organic fertilisation (Dix10) showed an increase in the total organic acid concentration from November to December in both external (54%) and internal (339%) leaves, the effect being particularly marked in the latter. However, the concentration decreased from November to January in both types of leaves (by 60% in external leaves and 8.6% in internal leaves).

CONCLUSIONS

The use of a conventional fertilisation regime with nitrogen, boron or sulfur improved the growth of *B. oleracea* var. *costata* in comparison with organic fertilisation, without affecting the organic acid profile. However, total organic acid concentration was observed to decrease under all agronomic practices tested for consecutive harvests with a time difference of 2 months. A 1 month difference, counting from the first harvest, markedly increased the content of organic acids under organic fertilisation.

Leaf age influenced the quantitative composition of organic acids. External leaves of plants grown under the same fertilisation conditions and collected at the same time usually presented higher total organic acid levels. Internal leaves presented higher citric acid relative contents, while malic acid was present in higher proportions in external leaves.

This work has extended the characterisation of organic acids in tronchuda cabbage, increasing our knowledge of this species grown under different conditions. Conventional fertilisation leads to the production of tronchuda cabbage with higher organic acid contents. This can help producers and consumers to choose products with increased levels of organic acids, which are known to be health-promoting compounds.

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4.8. Antioxidative properties of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) external leaves against DPPH, superoxide radical, hydroxyl radical and hypochlorous acid

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Antioxidative properties of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) external leaves against DPPH, superoxide radical, hydroxyl radical and hypochlorous acid

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Abstract

The ability of the aqueous extract of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) external leaves to act as a scavenger of DPPH$^{\cdot}$ and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid) was investigated. A phytochemical study was also undertaken, and thirteen phenolic compounds and five organic acids were identified and quantified. Tronchuda cabbage extracts exhibited antioxidant capacity in a concentration-dependent manner in all assays, although some pro-oxidant effect was also noticed. The samples with higher phenolic and organic acid contents displayed the major antioxidant potentials.
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Keywords: Tronchuda cabbage; *Brassica oleracea* L. var. *costata* DC; DPPH; Superoxide radical; Hydroxyl radical; Hypochlorous acid; Phenolics; Organic acids

1. Introduction

Reactive oxygen species (ROS) are generated by many redox processes that normally occur in the metabolism of aerobic cells. If not eliminated, ROS can attack important biological molecules, such as lipids, proteins, enzymes, DNA and RNA (Jung, Park, Chung, Kim, & Choi, 1999; Pietta, Simonetti, & Mauri, 1998; Yen & Chen, 1995). Although the human body possesses many defence mechanisms against oxidative stress, including antioxidant enzymes and non-enzymatic compounds, an excess of free radicals can go out of control, the organism being unable to scavenge all ROS (Halliwell, Aeschbach, Löliger, & Aruoma, 1995; Miyake et al., 2000; Sies, 1993; Tseng et al., 1997). Their excess has been implicated in the development of chronic diseases, such as cancer, arteriosclerosis, nephritis, diabetes mellitus, rheumatism, ischemic and cardiovascular diseases and also in the ageing process (Behl & Moosmann, 2002; Gyamfi, Yonamine, & Aniya, 1999; Pulido, Bravo, & Saura-Calixto, 2000). Oxidative stress can also play an important role in the development of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (Behl & Moosmann, 2002; Coulson, Siobhan, Cathal, Passmore, & Johnston, 2004). There is convincing epidemiological evidence that the

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consumption of fruits and vegetables is, in general, beneficial for health, due to the protection provided by the antioxidant phytonutrients contained in them (Guthrie & Kurowska, 2001). Especially vitamin C, vitamin E, carotenoids and dietary flavonoids can play important roles in human nutrition, and foods rich in these compounds should be involved in an optimal diet (du Toit, Volsteedt, & Apostolides, 2001).

Brassicaceae vegetables are reported to reduce the risks of some cancers, especially due to their contents of glucosinolates and derived products (Beecher, 1994; Park & Pezzuto, 2002; Stoewsand, 1995). Flavonoids and other phenolics are also considered to contribute to this capacity (Galati & O'Brien, 2004; Hertog, Hollman, & Van de Putte, 1993; Hollman, Hertog, & Katan, 1996). The antioxidant activity of some *Brassica oleracea* varieties has already been investigated. Cauliflower has been assayed for the ability to scavenge $DPPH^{\cdot}$ and $ABTS^{\cdot+}$, as well as for ferric reducing efficiency and ability to inhibit lipid peroxidation (Llorach, Espín, Tomás-Barberán, & Ferreres, 2003). The oxygen radical absorbance capacity values have been measured in broccoli (Kurilich, Jeffery, Juvik, Wallig, & Klein, 2002; Ninfali & Bacchiocca, 2003). To find the antiradical activity of white cabbage, hydroxyl radical was used (Racchi et al., 2002).

Tronchuda cabbage (*B. oleracea* L. var. *costata* DC) is consumed throughout the world, being widely used in the preparation of soups. Its external leaves are rather bitter and tough and are usually prepared by boiling. The analysis of phenolics in tronchuda cabbage external leaves has already been done and fourteen phenolic compounds were identified and quantified (Ferreres et al., 2005). However, as far as we know, nothing has been reported about the antioxidant properties of tronchuda cabbage.

The aim of the present study was to evaluate the antioxidant potential of four tronchuda cabbage external leaves aqueous extracts, since this is the most common use of the species. Therefore, the capacity to act as scavenger of $DPPH^{\cdot}$ and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid) was investigated. In order to define the phenolic and organic acid compositions of the extracts, we caried out, respectively, HPLC/diode array (HPLC/DAD) and HPLC/UV analysis. A possible relationship between chemical composition and antioxidant potential was established.

2. Materials and methods

2.1. Standards and reagents

The standards were from Sigma (St. Louis, MO, USA) and from Extrasynthése (Genay, France). Methanol and formic acid were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA). DPPH, xanthine, xanthine oxidase (XO) grade I from buttermilk (EC 1.1.3.22), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), anhydrous ferric chloride ($FeCl_3$), ethylenediaminetetraacetic acid disodium salt (EDTA), ascorbic acid, trichloroacetic acid, thiobarbituric acid, deoxyribose, sodium hypochlorite solution with 4% available chlorine (NaOCl) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. (St. Louis, USA).

2.2. Plant material and sampling

Tronchuda cabbages grown by different agronomic practices and collected in two periods (Table 1) were studied. These samples were chosen among previously studied ones (Ferreres et al., 2005) because of their distinct phenolic composition. After harvesting, plants were immediately transported to the laboratory, where external and internal leaves were separated. Samples were stored in a freezer at −20 °C.

2.3. Sample preparation

Tronchuda cabbage external leaves extracts were prepared by pouring 600 ml of boiling water on to 30 g of plant material. The mixture was boiled for one hour and then filtered through a Büchner funnel. The resulting extract was then lyophilised in a Labconco 4.5 apparatus (Kansas City, MO). The yields of the lyophilised extracts of samples A, B, C and D were 1.19, 1.07, 1.91 and 0.98 g, respectively. The lyophilised extracts were kept in a desiccator, in the dark.

2.4. HPLC analysis of organic acids

The separation was carried out as previously reported (Silva, Andrade, Mendes, Seabra, & Ferreira, 2002) with an analytical HPLC unit (Gilson), using an ion exclusion column Nucleogel® Ion 300 OA (300 × 7.7 mm) column. Detection was performed with a UV detector set at 214 nm.

Table 1
Characterization of tronchuda cabbage samples

Date of collection	Sample	Cultivation procedure
November 2002	A	Organic
	B	Conventional
December 2002	C	Organic
	D	Conventional

Organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards.

2.5. HPLC analysis of phenolics

Twenty microlitres of each extract were analysed using a HPLC unit (Gilson) and a Spherisorb ODS2 reversed-phase (Waters, Milford, USA) column (250 × 4.6 mm, 5 μm particle size) (Ferreres et al., 2005). The solvent system was a mixture of formic acid 5% (A) and methanol (B), with a flow rate of 1 ml min^{-1}, and the gradient was as follows: 0 min – 10% B, 25 min – 20% B, 40 min – 50% B, 45 min – 50% B, 46 min – 90% B, 50 min – 90% B, 55 min – 100% B, 58 min – 100% B, 60 min – 10% B. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200–400 nm, and chromatograms were recorded at 330 nm. The data were processed on a Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. With the exception of kaempferol 3-*O*-glucoside, which was quantified as itself, the identified compounds were quantified as kaempferol 3-*O*-rutinoside, if not commercially available.

2.6. DPPH$^{\cdot}$ scavenging activity

The antiradical activity of the extracts was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc.), by monitoring the disappearance of DPPH$^{\cdot}$ at 515 nm, according to a described procedure (Silva et al., 2004). For each extract, a dilution series (five different concentrations) was prepared in a 96 well plate. The reaction mixtures in the sample wells consisted of extract and DPPH radical dissolved in methanol. Three experiments were performed in triplicate.

2.7. Evaluation of superoxide radical scavenging activity

2.7.1. General

Antiradical activity was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc), by monitoring the effect of the lyophilised extracts on the $O_2^{\cdot -}$-induced reduction of NBT at 562 nm.

2.7.2. Non-enzymatic assay

Superoxide radicals were generated by the NADH/PMS system according to a described procedure (Valentão et al., 2001). All components were dissolved in phosphate 19 mM buffer, pH 7.4. Three experiments were performed in triplicate.

2.7.3. Enzymatic assay

Superoxide radicals were generated by the xanthine/xanthine oxidase (X/XO) system, following a described procedure (Valentão et al., 2001). Xanthine was dissolved in 1 μM NaOH and subsequently in phosphate 50 mM buffer with 0.1 mM EDTA, pH 7.8, xanthine oxidase in 0.1 mM EDTA and the other components in 50 mM phosphate buffer with 0.1 mM EDTA, pH 7.8. Three experiments were performed in triplicate.

2.8. Effect on xanthine oxidase activity

The effect of the lyophilised extracts on xanthine oxidase activity was evaluated by measuring the formation of uric acid from xanthine in a double beam spectrophotometer (Helios α, Unicam), at room temperature. The reaction mixtures contained the same proportion of components as in the enzymatic assay for superoxide radical scavenging activity, except NBT, in a final volume of 600 μl. The absorbance was measured at 295 nm for 2 min. Three experiments were performed in triplicate.

2.9. Hydroxyl radical assay

The deoxyribose method for determining the scavenging effect of the aqueous extracts on hydroxyl radicals was performed according to a described procedure (Valentão et al., 2002) in a double beam spectrophotometer (Helios α, Unicam). Reaction mixtures contained ascorbic acid, $FeCl_3$, EDTA, H_2O_2, deoxyribose and lyophilised extracts. All components were dissolved in 10 mM KH_2PO_4–KOH buffer, pH 7.4. This assay was also performed either without ascorbic acid or EDTA, in order to evaluate the extracts, pro-oxidant and metal chelation potentials, respectively. Three experiments were performed in triplicate.

2.10. Hypochlorous acid scavenging activity

The inhibition of hypochlorous acid-induced 5-thio-2-nitrobenzoic acid (TNB) oxidation to 5,5′-dithiobis(2-nitrobenzoic acid) was performed according to a described procedure (Valentão et al., 2002), in a double beam spectrophotometer (Helios α, Unicam). Hypochlorous acid and TNB were prepared immediately before use. Scavenging of hypochlorous acid was ascertained by using lipoic acid as a reference scavenger, which inhibited TNB oxidation in a concentration-dependent manner. Three experiments were performed in triplicate.

3. Results and discussion

3.1. Identification and quantification of phenolic compounds by HPLC/DAD

The aqueous lyophilised extracts of tronchuda cabbage external leaves presented different phenolic compositions. In sample D, only three compounds were detected: kaempferol 3-*O*-sophorotrioside, kaempferol 3-*O*-(sinapoyl)-sophoroside and kaempferol 3-*O*-(feruloyl)-sophorotrioside (Table 2). In sample C, besides kaempferol 3-*O*-(feruloyl)-sophorotrioside, we identified kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside, kaempferol 3-*O*-(feruloyl)-sophoroside, kaempferol 3-*O*-sophoroside and kaempferol 3-*O*-glucoside (Table 2). Sample B showed all the compounds described above and kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside, kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside and kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside (Table 2). Sample A exhibited a chemical profile composed of the 12 above-mentioned kaempferol derivatives and kaempferol 3-*O*-sophoroside-7-*O*-sophoroside (Fig. 1 and Table 2). These compounds have already been identified in tronchuda cabbage external leaves methanolic extracts (Ferreres et al., 2005).

Sample A was the one with the highest amount of phenolics (ca. 1231 mg/kg) (Table 2), kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside being the main compound (26% of total identified compounds). Sample B had a lower phenolics content (ca. 32 mg/kg) (Table 2), in which kaempferol 3-*O*-sophoroside was the compound present in the highest amount (32%). In sample D, kaempferol 3-*O*-(feruloyl)-sophorotrioside was the major kaempferol derivative, while sample C exhibited only vestigial amounts of phenolics (Table 2).

As previously observed (Ferreres et al., 2005), when considering the evolution during winter, a decrease of the total phenolics content was noticed between November and December, which is more evident in samples from organic culture. The lowest level of total phenolics, observed in December in a sample from organic production, may be explained by the commitment of tronchuda cabbage cells to morphogenic developmental pathways, since those cabbages presented more developed leaves than those of conventional culture in the same period. In addition, samples from organic culture exhibited higher total phenolics content than those from conventional practices, collected in the same period, with the exception of the samples from December. This might be due to the interference of the mineral fertilizers and/or pesticides, used in conventional practices, in the biosinthetic pathway of flavonoids, decreasing phenolic amounts (Ferreres et al., 2005).

3.2. Identification and quantification of organic acids by HPLC/UV

Tronchuda cabbage external leaves had a chemical profile composed of five identified organic acids: citric, ascorbic, malic, shikimic and fumaric acids (Fig. 2). These compounds are reported for the first time in tronchuda cabbage. As observed for phenolic compounds, sample A exhibited the highest content of organic acids (ca. 55 g/kg) and sample C the lowest one (ca. 20 g/kg) (Table 3). In samples A, B and C, malic acid was the main compound, ranging from 37% to 78% of total identified organic acids, followed by citric acid (16–36%) (Table 3). Sample D showed a distinct profile, in

Table 2
Phenolic composition of tronchuda cabbage external leaves (mg of phenolic compound kg^{-1} of lyophilised extract)[a]

Compounds[b]	Samples							
	A		B		C		D	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1 + **2** (RT 24.0, 24.2 min)	73.1	2.1	nq	–	nd	–	nd	–
3 + **4** (Rt 25.8, 26.0 min)	237	5.2	8.2	0.5	nq	–	nd	–
5 (RT 27.8 min)	46.3	6.9	nd	–	nd	–	nd	–
6 (RT 30.7 min)	119	5.9	nq	–	nd	–	nd	–
7 (RT 31.2 min)	323	7.0	2.5	0.1	nd	–	nd	–
8 (RT 36.5 min)	26.1	0.1	nq	–	nd	–	nq	–
9 (RT 37.4 min)	nq	–	2.6	0.0	nd	–	nq	–
10 (RT 38.5 min)	85.8	1.5	7.4	0.0	nq	–	5.9	0.5
11 (RT 39.3 min)	99.8	1.8	1.3	0.1	nq	–	nd	–
12 (RT 39.7 min)	221	5.2	10.6	0.1	nq	–	nd	–
13 (RT 44.1 min)	nq	–	nq	–	nq	–	nd	–
Σ	1231.2		32.6		–		5.9	

[a] Results are expressed as means of three determinations. SD, standard deviation, ∑, sum of the determined phenolic compounds.
[b] Identity of compounds as in Fig. 1.

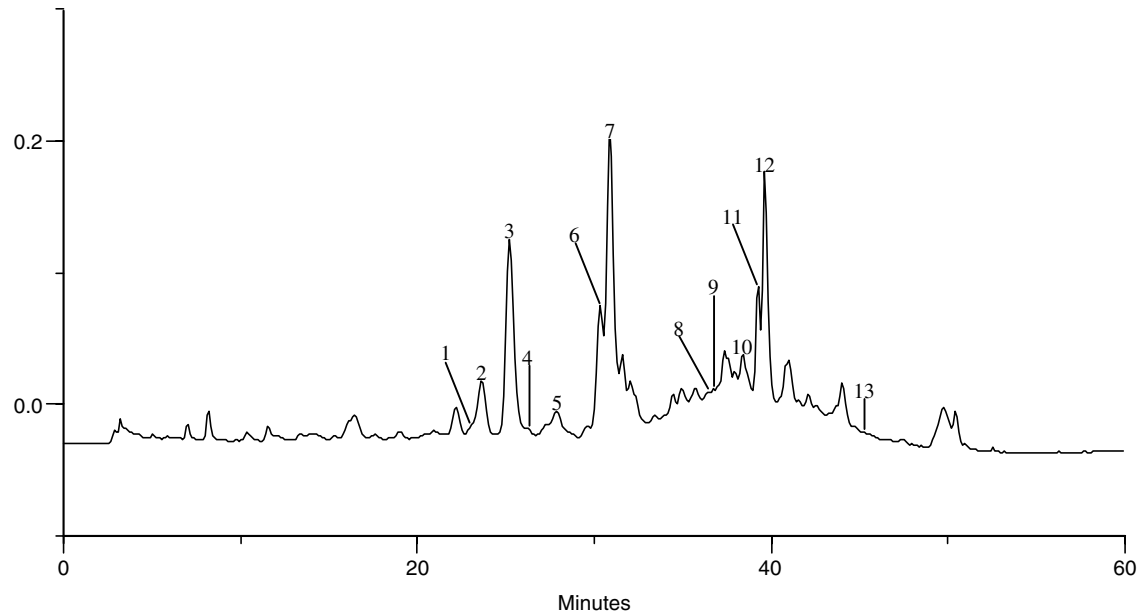

Fig. 1. HPLC phenolic profile of tronchuda cabbage external leaves. Detection at 330 nm. Peaks: (1) kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside; (2) kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside; (3) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (4) kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside; (5) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside; (6) kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside; (7) kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside; (8) kaempferol 3-*O*-sophorotrioside; (9) kaempferol 3-*O*-(sinapoyl)-sophoroside; (10) kaempferol 3-*O*-(feruloyl)-sophorotrioside; (11) kaempferol 3-*O*-(feruloyl)-sophoroside; (12) kaempferol 3-*O*-sophoroside; (13) kaempferol 3-*O*-glucoside.

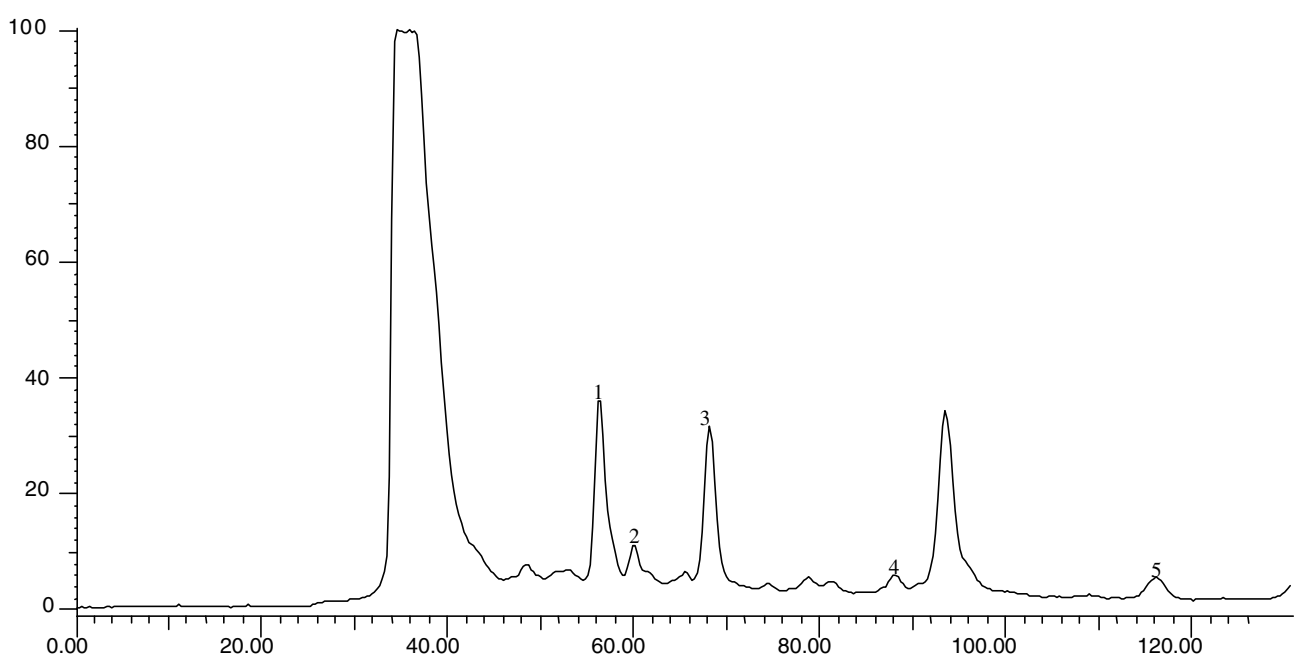

Fig. 2. HPLC organic acid profile of tronchuda cabbage external leaves. Detection at 214 nm. Peaks: (1) citric acid; (2) ascorbic acid; (3) malic acid; (4) shikimic acid; (5) fumaric acid.

which ascorbic acid was the acid present in highest amounts representing 44% of total compounds, followed by malic acid (39%) (Table 3). In all samples, shikimic and fumaric acids were minor compounds (Table 3).

3.3. Antioxidant activity

Antioxidant capacities of tronchuda cabbage external leaves aqueous lyophilised extracts are shown in Table 4

Table 3
Organic acids in tronchuda cabbage external leaves (mg of organic acid kg^{-1} of lyophilised extract)[a]

Compound[b]	Samples							
	A		B		C		D	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1 (RT 56.7 min)	18464	52.2	9101	172	3312	185	6770	3.0
2 (RT 60.1 min)	10609	12.1	2937	2.8	1028	146.3	17343	206
3 (RT 68.6 min)	20506	57.3	13385	171	15472	286.9	15274	222
4 (RT 88.9 min)	24.1	0.2	17.3	2.0	31.7	1.3	34.0	3.8
5 (RT 116.8 min)	5336	115	27.7	0.1	111	0.0	58.0	1.0
Σ	54939		25468		19954		39479	

[a] Results are expressed as means of three determinations: SD, standard deviation, $\sum$, sum of the determined organic acids.
[b] Identity of compounds as in Fig. 2.

Table 4
IC_{25} values (mg ml^{-1}) of tronchuda cabbage external leaves in antiradical assays

Samples	DPPH IC_{25}	$O_2^{\bullet-}$ (non-enzymatic) IC_{25}	$O_2^{\bullet-}$ (enzymatic) IC_{25}	$^{\bullet}OH$ IC_{25}
A	0.681	0.102	0.047	0.019
B	1.66	0.045	0.139	0.045
C	2.32	0.362	0.311	0.223
D	1.14	0.146	0.101	0.018

and Figs. 3–6. The DPPH$^{\bullet}$ test is a non-enzymatic method currently used to provide basic information on the ability of extracts to scavenge free radicals. Reduction of DPPH$^{\bullet}$ by an antioxidant results in a loss of absorbance at 515 nm (Fukumoto & Mazza, 2000). Our results indicated that sample A was the one with the highest antioxidant potential, measured by the DPPH assay, followed by samples D and B, respectively. Sample C exhibited the lowest scavenging capacity (Table 4 and Fig. 3). The effects observed were concentration-dependent.

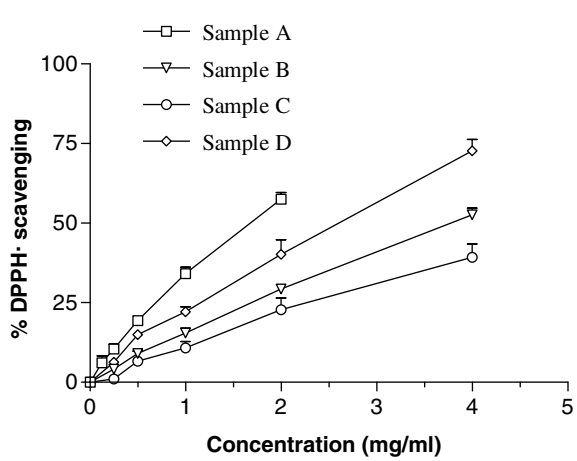

Fig. 3. Effect of tronchuda cabbage external leaves on DPPH$^{\bullet}$ reduction. Values show means ± SE from 3 experiments performed in triplicate.

Superoxide radical is produced in vivo by activated phagocytes, by electron leakage from the mitochondrial electron transport chain (Halliwell, 1991) and in the conversion of xanthine to uric acid (Bast, Haenen, & Doelman, 1991). The reactivity of this radical is limited but it is considered to be toxic. In fact, much of the molecular damage that can be done by superoxide radical is due to its conversion into much more reactive species, namely hydroxyl radical and peroxynitrite (Halliwell et al., 1995).

In the present work, the lyophilised extracts of tronchuda cabbage external leaves exhibited superoxide radical-scavenging activity using the X/XO system in a concentration-dependent manner (Fig. 4(a)), sample A being the most active (Table 4). Bearing in mind that an inhibitory effect on the enzyme itself would also lead to a decrease of NBT reduction (Halliwell et al., 1995), the effect of the extracts on XO activity was checked. For this purpose a control experiment was performed evaluating the effect of tronchuda cabbage lyophilised extracts on the metabolic conversion of xanthine to uric acid. The results revealed that samples A, C and D had a weak inhibitory effect on XO, while sample B presented higher inhibitory activity (IC_{20} at 184 μg/ml) (Fig. 4(b)), so it was not possible to show a clear-cut scavenging effect on superoxide radical. In view of clarification, the effect of the aqueous extracts on superoxide radical, generated by a chemical system composed of PMS, NADH and oxygen, was also evaluated. Under the assay conditions, all the samples were able to scavenge $O_2^{\bullet-}$ in a concentration-dependent way (Fig. 4(c)), in which sample B showed the strongest scavenging activity (Table 4).

Hydroxyl radical is the most reactive radical known: it can attack and damage almost every molecule found in living cells. Reactions of $^{\bullet}OH$ include its ability to interact with the purine and pyrimidine bases of DNA. It can also abstract hydrogen atoms from biological molecules, including thiols, leading to the formation of sulfur radicals able to combine with oxygen to generate oxysulfur radicals, a number of which damage biological molecules (Halliwell, 1991). The best-characterised

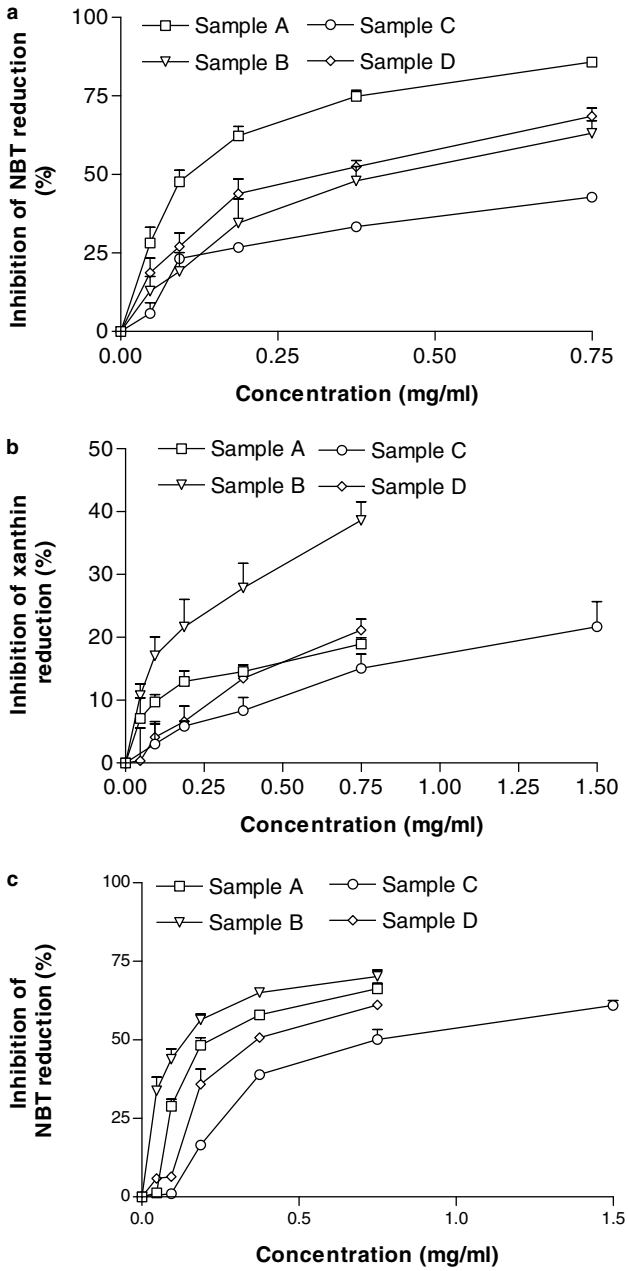

Fig. 4. Effect of tronchuda cabbage external leaves on: (a) NBT reduction induced by superoxide radical generated in a X/XO system; (b) on XO activity; (c) NBT reduction induced by superoxide radical generated in a NADH/PMS system. Values show means ± SE from 3 experiments performed in triplicate.

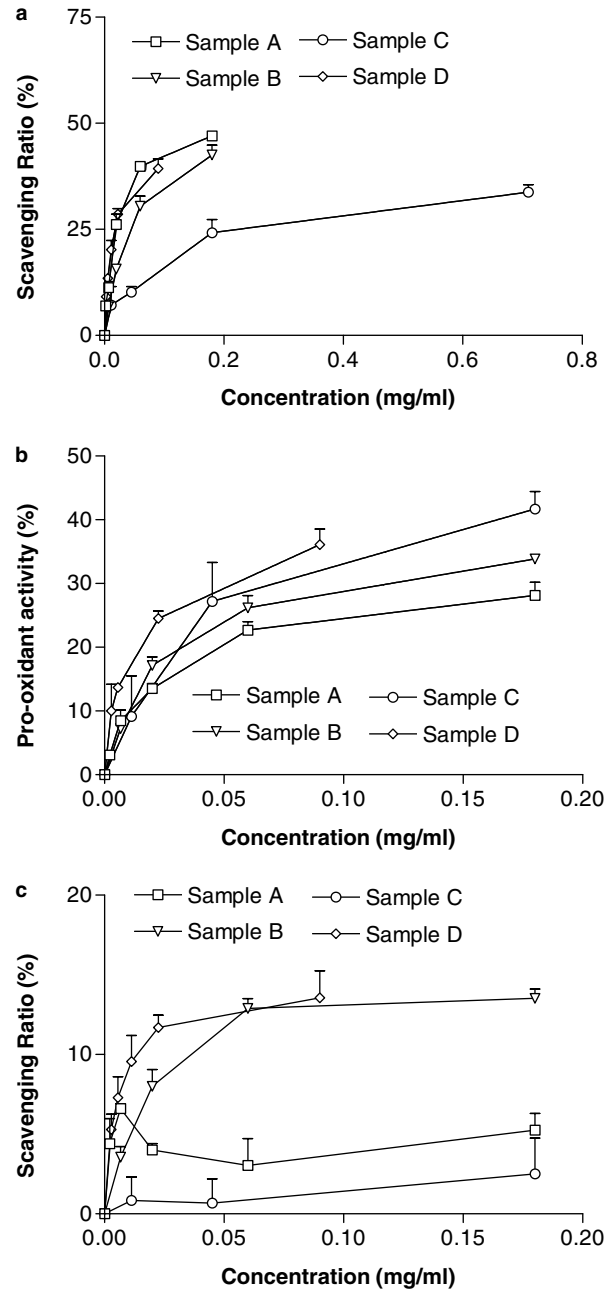

Fig. 5. Effect of tronchuda cabbage external leaves: (a) non-specific hydroxyl radical-scavenging activity; (b) pro-oxidant activity (-AA); (c) specific hydroxyl radical-scavenging (-EDTA). Values show means ± SE from 3 experiments performed in triplicate.

biological damage caused by hydroxyl radical is its capacity to stimulate lipid peroxidation, which occurs when ·OH is generated close to membranes and attacks the fatty acid side chains of the membrane phospholipids (Halliwell, 1991).

Hydroxyl radicals are produced in vivo by Fenton-type reactions, in which transition metals (e.g., iron) reduce hydrogen peroxide. Reducing agents, such as ascorbic acid, can accelerate ·OH formation by reducing Fe^{3+} ions to Fe^{2+} (Puppo, 1992). Deoxyribose is degraded into malonaldehyde on exposure to hydroxyl radicals generated by Fenton systems. If the resulting mixture is heated under acid conditions, malonaldehyde may be detected by its ability to react with thiobarbituric acid to form a pink chromogen (Halliwell, Gutteridge, & Aruoma, 1987). In the work herein, tronchuda cabbage lyophilised extracts exhibited scavenging activity for hydroxyl radical in a concentration dependent manner (Fig. 5(a)), with samples A and D presenting the highest capacity (Table 4). Sample C exhibited minor ability.

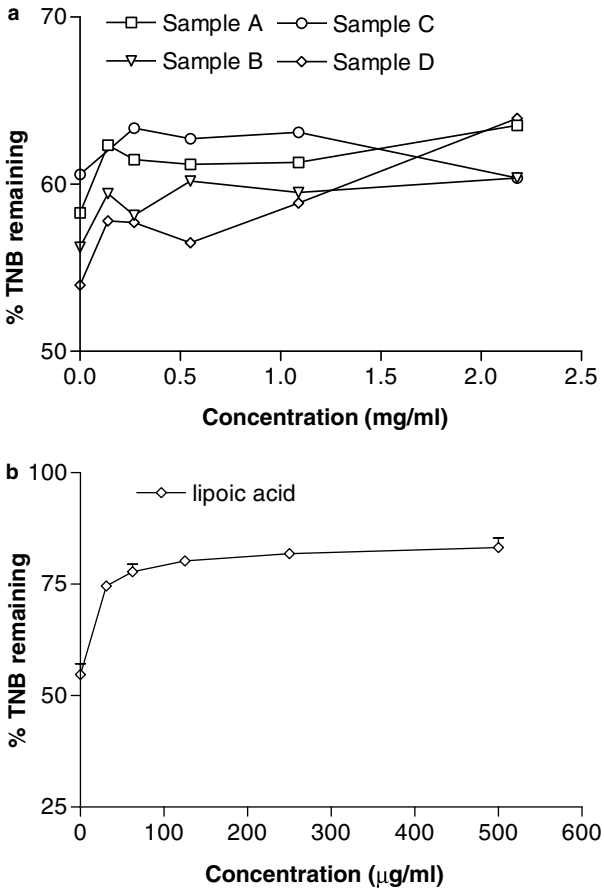

Fig. 6. Effects of tronchuda cabbage external leaves (a) and lipoic acid (b) on the oxidation of TNB by HOCl. The amount of TNB unchanged after incubation is calculated and expressed as percentage of the initial value. Values show means ± SE from 3 experiments performed in triplicate.

However, some compounds have the ability to cause redox cycling of the metal ion required for hydroxyl generation, thus increasing the radical production, exhibiting pro-oxidant activity (Li & Xie, 2000). In order to evaluate the pro-oxidant potential of the extracts, we omitted ascorbic acid. As can be seen in Fig. 5(b), all samples were a very effective substitute for ascorbic acid. Thus, these extracts may act as concentration-dependent pro-oxidants, that from sample D being the most active.

Damage to deoxyribose also occurs if the Fe^{3+}-ascorbate-H_2O_2-induced generation of hydroxyl radicals is performed in the absence of EDTA, since omission of the chelator allows iron ions to bind directly to the sugar. Under such conditions, compounds inhibit deoxyribose degradation, not by reacting with hydroxyl radicals, but because they present ion-binding capacity and can withdraw the iron ions and render them inactive or poorly active in Fenton reactions (Payá, Halliwell, & Hoult, 1992). The assay performed in the absence of EDTA showed that extracts from samples B and D were, to some extent, able to chelate iron ions in a concentration-dependent manner, while those from samples A and C were practically ineffective (Fig. 5(c)).

Reactive oxygen species produced in vivo by activated phagocytic cells include HOCl. Hypochlorous acid is produced in the organism at sites of inflammation by the oxidation of Cl^- ions, catalysed by neutrophil-derived myeloperoxidase, in the presence of H_2O_2 (Aruoma, Halliwell, Hoey, & Butler, 1989). HOCl is a powerful oxidant, which reacts readily with many biologically important molecules. Thiol groups are easily oxidized by HOCl (Ching, De Jong, & Bast, 1994). HOCl damages and induces target cell lysis, caused by sulfhydryl oxidation in plasma membrane proteins (Cochrane, 1991), inactivates α_1-antiprotease, activates collagenase and gelatinase, depletes antioxidant vitamins, such as ascorbic acid, and inactivates antioxidant enzymes such as catalase (Payá et al., 1992; Visioli, Bellomo, & Galli, 1998).

In the present assay, HOCl induces the oxidation of TNB ($\lambda_{max} = 412$ nm) into DTNB ($\lambda_{max} = 325$ nm) (Künzel, Zee, & Ijzerman, 1996). An HOCl scavenger inhibits the oxidation of TNB by this species. When the antioxidant protection against damage by HOCl was evaluated, tronchuda cabbage lyophilised extracts exhibited a very weak antioxidant protective activity, with the exception of sample D, which showed some concentration dependent capacity (Fig. 6(a)). In the assayed conditions, lipoic acid was used as a reference compound, which scavenged HOCl effectively in a concentration- dependent manner, presenting a protective effect of 83% at 500 μg/ml (Fig. 6(b)).

In general terms, and according to the results obtained in all assays, sample A proved to be the one with highest antioxidant potential. This is not surprising, considering that it is the sample with the highest content of both phenolics and organic acids (Tables 2 and 3), which are known to have antioxidant activity (Madhavi, Singhal, & Kulkarni, 1996). In addition, sample A has a high amount of acylated flavonols, namely caffeoyl derivatives (Table 2). These compounds are reported to have high scavenging ability due to the presence of an *O*-dihydroxy structure in the caffeoyl moiety, which confers great stability to the radical form and participates in the electron delocalisation (Braca et al., 2003). The presence of high contents of caffeic acid derivatives may also contribute to its lower pro-oxidant effect, because of the presence of a catechol ring (Galati, Sabzevari, Wilson, & O' Brien, 2002).

In opposition, sample C displayed the lowest antioxidant capacity, which can be ascribed to the presence of only vestigial amounts of phenolic compounds and to the minor organic acid content. Furthermore, this sample exhibited the lowest ascorbic acid content, when compared with the other samples. In spite of this, sample C showed a pro-oxidant effect, which might be related to the presence of other compounds in the extract.

The great antioxidant activity presented by sample D may be attributed to its major content of ascorbic acid. However, the high amount of this organic acid can also explain the pro-oxidant effect observed in the deoxyribose assay performed in the absence of ascorbic acid.

Although the antiradical activity of phenolic compounds and organic acids is already known, it remains uncertain how a complex mixture, obtained from plant extracts, functions against reactive oxygen species, because other compounds of the mixture may potentiate or prevent the expected activity. In fact, this study indicates that phenolics and organic acids were not correlated with antioxidant capacity of the aqueous lyophilised extracts.

In conclusion, considering the results obtained and regarding the presence of several phenolics and organic acids in the aqueous lyophilised extracts of tronchuda cabbage external leaves, the scavenging activities observed against DPPH$^{\bullet}$, superoxide radical, hydroxyl radical and hypochlorous acid are most probably due to the presence of these compounds, which contribute to the protective effects observed in the work herein. As far as we know, this is the first study concerning the antioxidant activity of tronchuda cabbage, and indicates that its external leaves can be used as good sources of antioxidants in our diet. This may be relevant in the prevention of diseases in which free radicals are involved.

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4.9. Free amino acids of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC): Influence of leaf position (internal or external) and collection time"

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Free Amino Acids of Tronchuda Cabbage (*Brassica oleracea* L. Var. *costata* DC): Influence of Leaf Position (Internal or External) and Collection Time

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The free amino acid profile of 18 samples of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) leaves, harvested at three different months, was determined by HPLC/UV−vis. The tronchuda cabbage leaves total free amino acid content varied from 3.3 to 14.4 g/kg fresh weight. Generally, arginine was the major compound, followed by proline, threonine, glutamine, cysteine, and glutamic acid. This study indicates that free amino acids are not similarly distributed: in external leaves, proline and arginine were the major free amino acids, while in internal ones, arginine was the main free amino acid, followed by threonine, glutamine, and cysteine. Significant differences were observed for valine, proline, arginine, leucine, cysteine, lysine, histidine, and tyrosine contents. The levels of some free amino acids were significantly affected by the collection period. In external leaves, this occurred with glutamic acid, serine, valine, leucine, cysteine, and ornithine contents, while in internal leaves, it occurred with aspartic acid, arginine, and total contents.

KEYWORDS: ***Brassica oleracea*** L. var. *costata* DC; tronchuda cabbage; internal and external leaves; free amino acids

INTRODUCTION

Experimental, clinical, and population studies confirmed the benefit of diets that are rich in fruits and vegetables for the prevention of cardiovascular diseases, cancer, hypertension, diabetes, and obesity (*1*). Brassicaceae plants represent one of the major vegetable crops grown, worldwide, constituting an important part of a well-balanced diet (*2*), such as a Mediterranean one. In Europe, owing to the availability of local markets, inexpensiveness, and consumer preference, cruciferous vegetables, such as cabbage, are among the most important dietary vegetables consumed (*1*). Several epidemiological studies report an inverse correlation between consumption of Brassicaceae and risk of cancer (*1*). The cancer preventive properties mainly were attributed to the glucosinolates and their derived products; however, phenolic compounds, such as flavonoids, also contribute to these capacities (*3*).

Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) is especially popular in Portugal, having a determinant role in the Portuguese diet and agricultural systems (*2*). It is a hardy crop that is high yielding, less susceptible to pests and diseases, well-adapted to a wide range of climates, and generally grown with little or no agrochemical input. The cabbage plant resembles a thick-stemmed collard with large floppy leaves. The leaves are close together, round, smooth, and slightly notched at the margins and are eaten raw or cooked. The internal and external leaves are considerably different with regard to organoleptic characteristics, which may influence the consumer's preferences. Internal leaves are pale yellow, tender, and sweeter than external leaves, which are dark green (*3*).

Consumers have increased their awareness concerning food composition, and further comprehensive information has been demanded, beyond that available in food composition tables. In such tables, the amino acid composition is limited and mostly given as both free and bound amino acids. Since free amino acids are involved in secondary plant metabolism and in the biosynthesis of compounds, such as glucosinolates and phenolics, which directly or indirectly play an important role in plant−environment interaction and human health, free amino acid profile determination becomes more relevant (*4*).

In the past few years, the *B. oleracea* var. *costata* chemical composition and antioxidant potential were studied by our

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research group (*2, 3, 5–8*). Regarding phenolic composition, external leaves showed high levels of complex flavonol glycosides, while the internal leaves exhibited both flavonol glycosides and hydroxycinnamic acid derivatives (*3, 5*). The organic acid profile and antioxidant capacity of external and internal leaves against DPPH and reactive oxygen species (superoxide and hydroxyl radicals and hypochlorous acid) also were described, with external leaves exhibiting a higher antioxidant potential (*5, 6*). The influence of two fertilization regimens (conventional and organic practices) on the amounts of organic acids and phenolic compounds of tronchuda cabbage also was reported (*8*). The results obtained in this study indicated that, in a general way, tronchuda cabbage from organic cultures presents a higher phenolic content than that from a conventional culture (*8*).

In this work, we analyzed the free amino acid composition in tronchuda cabbage leaves. We also clarified as to whether there were any differences between internal and external leaves and harvesting months. By these means, it is possible to know if it is a material or a period in which the production of amino acids is clearly higher, which would constitute a nutritional advantage. For this purpose, a methodology based on free amino acid precolumn derivatization with dabsyl chloride and reversed-phase HPLC/UV−vis analysis was applied to tronchuda cabbage leaves.

MATERIALS AND METHODS

Standards and Reagents. All L-amino acid standards, dabsyl chloride reagent, sodium hydrogen carbonate, sodium dihydrogenphosphate, dimethylformamide, and triethylamine were from Sigma (St. Louis, MO). HPLC grade acetonitrile, ethanol, and phosphoric acid were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA). All other reagents were of analytical grade from several suppliers.

Samples. The experimental work was carried out in one field located in Bragança, NE Portugal (41°48′N, 6°44′W). Plant sowing occurred in the middle of June 2005, in a greenhouse (22 ± 2 °C, 80% humidity). Young plants were transplanted to the field at the end of August, spaced at 0.8 m × 0.5 m between and within rows, and all agricultural practices followed the traditional practices of the region with respect to fertilization regime, irrigation, and phytosanitary treatments.

Samples were collected on three occasions during the growth season: in mid-November 2005, mid-December 2005, and mid-January 2006. In each harvesting date, six plants were randomly collected in the field. All samples were collected in the morning, at the same hour. After harvesting, the plants were immediately transported to the laboratory, and external (older) and internal (younger) leaves were separated. Care was taken to choose plants and leaves of similar developmental stage: internal leaves, pale yellow and tender, were separated from the external ones, which were dark green and no longer actively expanding, although not yet senescent. Two hours (maximum) after their collection, the samples were frozen at −20 °C and then lyophilized (Labconco 4.5 Freezone apparatus, Kansas City, MO). The freeze-drying yield was ca. 14%. The freeze-dried samples were powdered and kept in a desiccator in the dark until they were subjected to aqueous extraction.

Extraction. Leaf extract was prepared by pouring 600 mL of boiling water on to ca. 30 g of plant material. The mixture was boiled for 1 h and then filtered through a Buchner funnel. The resulting extract was then lyophilized in a Labconco 4.5 apparatus (Kansas City, MO) and ca. 14 g of lyophilized extract was obtained, which was afterward kept in a desiccator, in the dark. Approximately 40 mg of each lyophilized extract was redissolved in 400 μL of 0.1 M HCl.

Derivatization Procedure. Dabsylation was achieved as reported by Silva et al. (*9*). Aliquots of 20 μL of standard solution (ca. 0.2 mg/mL each amino acid in 0.1 M HCl) or redissolved extract were diluted with 180 μL of the reaction buffer (0.15 M sodium hydrogen carbonate, pH 8.6 with NaOH). After thorough mixing on a vortex-mixer, 200 μL of the 12.4 mM dabsyl chloride reagent (in acetone) was added, and the vials were mixed again. The resulting solutions were incubated at 70 °C in a water bath for 15 min. The reaction was stopped by placing the vials in an ice bath for 5 min. A total of 400 μL of the dilution buffer [mixture of 50 mL of acetonitrile, 25 mL of ethanol, and 25 mL of 9 mM sodium dihydrogenphosphate 9; 4 % dimethylformamide; and 0.15% triethylamine (pH 6.55 with phosphoric acid)] was added, followed by thorough mixing and centrifugation (5 min, 5000 rpm). The clear supernatants were directly set for injection or stored at −20 °C.

Table 1. Equations for Regression Lines and Correlation Coefficients, Concentration Range of Linearity, and Detection Limits for Amino Acids

amino acid	equation[a]	linearity (μg/mL)	detection limit (μg/mL)
aspartic acid	$y = 1.42 \times 10^4 x$; $r = 0.99624$	1.25−10.0	0.85
glutamic acid	$y = 1.28 \times 10^4 x$; $r = 0.99994$	1.25−10.0	0.94
asparagine	$y = 1.47 \times 10^4 x$; $r = 0.98967$	0.59−4.68	0.81
glutamine	$y = 2.28 \times 10^4 x$; $r = 0.99795$	1.28−10.2	0.53
serine	$y = 3.22 \times 10^4 x$; $r = 0.99846$	1.26−10.1	0.38
threonine	$y = 1.48 \times 10^4 x$; $r = 0.99709$	1.27−10.2	0.81
glycine	$y = 8.25 \times 10^4 x$; $r = 0.99738$	1.29−10.3	0.15
alanine	$y = 5.75 \times 10^4 x$; $r = 0.99855$	1.27−10.1	0.22
valine	$y = 1.16 \times 10^4 x$; $r = 0.99823$	1.25−10.0	1.04
proline	$y = 4.82 \times 10^4 x$; $r = 0.99641$	1.25−10.0	0.25
arginine	$y = 5.95 \times 10^4 x$; $r = 0.99561$	1.26−10.1	0.20
isoleucine	$y = 6.19 \times 10^4 x$; $r = 0.99486$	1.29−10.3	0.20
leucine	$y = 4.46 \times 10^4 x$; $r = 0.99796$	1.28−10.2	0.28
tryptophan	$y = 3.32 \times 10^4 x$; $r = 0.99674$	1.25−10.0	0.36
phenylalanine	$y = 4.21 \times 10^4 x$; $r = 0.99446$	1.28−10.2	0.29
cysteine	$y = 1.05 \times 10^4 x$; $r = 0.99337$	1.28−10.2	1.15
ornithine	$y = 5.77 \times 10^4 x$; $r = 0.99826$	1.34−10.7	0.22
lysine	$y = 6.05 \times 10^4 x$; $r = 0.99876$	1.26−10.0	0.20
histidine	$y = 3.30 \times 10^4 x$; $r = 0.99606$	1.29−10.3	0.36
tyrosine	$y = 4.44 \times 10^4 x$; $r = 0.99816$	1.25−9.97	0.28

[a] *y*: peak area at 436 nm; *x*: μg of amino acid; and *r*: correlation coefficient.

HPLC/UV−vis Analysis. Dabsyl derivatives of free amino acids were separated on a Gilson HPLC unit, using a reversed-phase Spherisorb ODS2 column (25.0 cm × 0.46 cm; 5 μm particle size) (*9*). The solvent system consisted of 9 mM sodium dihydrogenphosphate, 4% dimethylformamide, and 0.15% triethylamine (pH 6.55 with phosphoric acid) (A) and 80% acetonitrile (B). Elution was performed at a flow rate of 1 mL/min, starting with 20% B until 7 min and installing a gradient to obtain 35% B at 35 min, 50% B at 45 min, and 100% B at 66 min. Detection was achieved with a UV−vis detector set at 436 nm. Free amino acid quantification was accomplished by the absorbance recorded in the chromatograms relative to external standards. Twenty microliters of the derivatized standard solutions or samples was injected.

Under the assay conditions described, a linear relationship between the concentration of amino acids and the absorbance at 436 nm was obtained in the tested range (**Table 1**). The correlation coefficient for the standard curves invariably exceeded 0.99 for all compounds. The detection limits were calculated as the concentration corresponding to 3 times the standard deviation of the background noise, and the values obtained were low, once they ranged from 0.15 to 1.15 μg/mL (**Table 1**).

Statistical Analysis. The results are expressed as mean values and standard error for the three sampling periods and type of leaf (internal and external). Analysis of variance followed by a Tukey test with $a = 0.05$ were performed to study the differences between harvesting times and leaf types for free amino acid composition using SAS v. 9.13.

RESULTS AND DISCUSSION

Amino acids are important for human nutrition and affect food quality, including taste, aroma, and color (*10*). When compared to other varieties, the *B. oleracea* var. *costata* leaf total free amino acid content was high, varying from 3.3 to 14.4 g/kg fresh weight (mean value of 7.9 g/kg fresh weight or 56.4 g/kg dry weight).

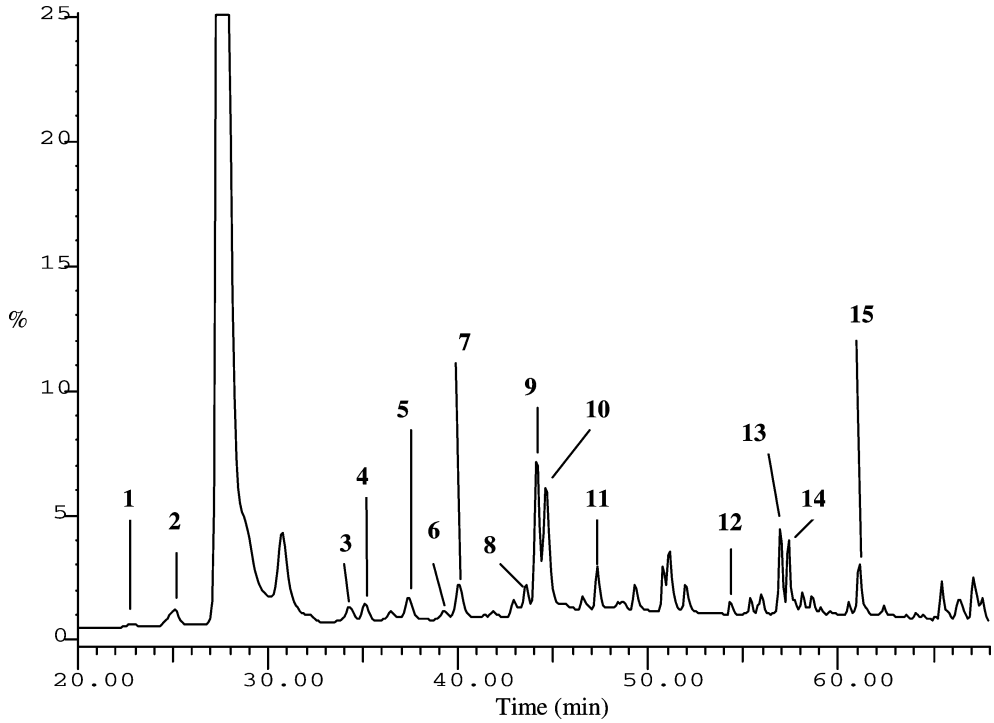

Figure 1. HPLC/UV−vis chromatogram of free amino acids in a tronchuda cabbage external leaf sample, collected in December. Detection was at 436 nm. (1) Aspartic acid; (2) glutamic acid; (3) glutamine; (4) serine; (5) threonine; (6) glycine; (7) alanine; (8) valine; (9) proline; (10) arginine; (11) leucine; (12) cysteine; (13) ornithine; (14) lysine; and (15) tyrosine.

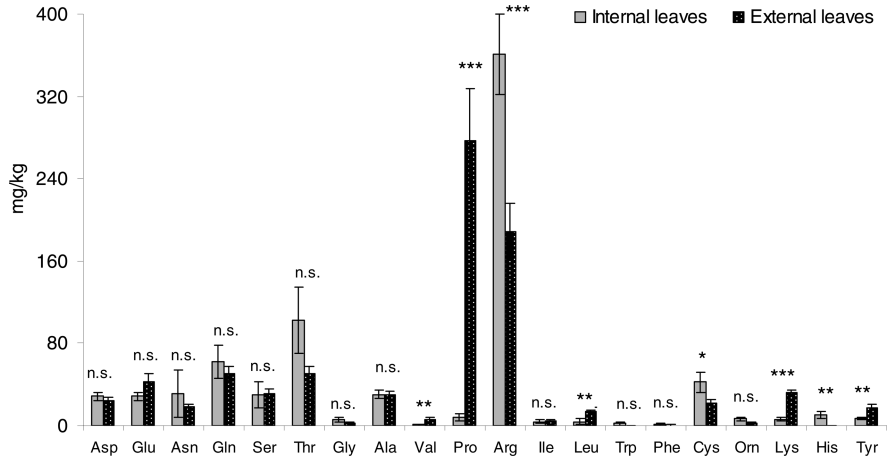

Figure 2. Quantitative free amino acid profile of tronchuda cabbage internal and external leaves samples, using all data combined (mean value, mg/kg). (asp) Aspartic acid; (glu) glutamic acid; (asn) asparagine; (gln) glutamine; (ser) serine; (thr) threonine; (gly) glycine; (ala) alanine; (val) valine; (pro) proline; (arg) arginine; (ile) isoleucine; (leu) leucine; (trp) tryptophan; (phe) phenylalanine; (cys) cysteine; (orn) ornithine; (lys) lysine; (his) histidine; and (tyr) tyrosine. *$0.01 < p \leq 0.05$ (significant difference); **$0.001 < p \leq 0.01$ (very significant difference); $p \leq 0.001$ (extremely significant difference); and n.s., nonsignificant difference.

Considering all tronchuda cabbage leaf samples, their free amino acid profile was highly dispersed among the 20 constituents (**Figure 1**). Nevertheless, in a general way, leaves were rich in terms of arginine (35.6% total free amino acid content); had medium values of proline, threonine, glutamine, cysteine, and glutamic acid (16.8, 8.9, 7.1, 4.7, and 4.6% total free amino acid content, respectively); presented low contents of alanine, serine, aspartic acid, lysine, asparagine, and tyrosine (4.0, 3.6, 3.4, 2.7, 2.4, and 1.7% total free amino acid content, respectively); and had very small proportions of the other eight free amino acids.

Arginine, the main compound, is a semiessential amino acid for humans, which is required to ensure that liver, joints, muscles (including the heart muscle), and skin are kept healthy. Arginine strengthens the immune system, promotes male fertility, and is involved in the regulation of many hormonal processes in the body (pituitary gland, pancreas, and human growth hormone). Arginine is semiessential because the body can usually produce enough amounts in normal circumstances, but when submitted to great physical stress or illness, more arginine is required. In addition, babies cannot produce arginine in their first few months. Arginine is also of great importance as an intermediary product in urea synthesis. This amino acid is present in all proteins at an average level of 3−6% (*10*).

Proline improves skin texture by aiding in the production of collagen and reduces its loss through the aging process. This nonessential amino acid works with ascorbic acid to promote healthy connective tissue. Proline also helps in the maintenance and healing of cartilage and the strengthening of joints, tendons, and muscles (including the heart muscle). Generally, proline is

Table 2. Free Amino Acid Composition of Tronchuda Cabbage External Leaf Samples (Mean ± SD) (g/kg Fresh Weight) at Different Collection Times[a]

amino acid (g/kg)	November 2005	December 2005	January 2006
aspartic acid	0.29 ± 0.08 a	0.23 ± 0.06 a	0.22 ± 0.03 a
glutamic acid	0.67 ± 0.16 a	0.42 ± 0.11 a,b	0.19 ± 0.05b
asparagine	0.17 ± 0.04 a	0.19 ± 0.05 a	0.18 ± 0.06 a
glutamine	0.61 ± 0.15 a	0.43 ± 0.10 a	0.48 ± 0.14 a
serine	0.51 ± 0.09 a	0.22 ± 0.03 b	0.20 ± 0.03 b
threonine	0.56 ± 0.14 a	0.50 ± 0.17 a	0.44 ± 0.08 a
glycine	0.03 ± 0.00 a	0.03 ± 0.01 a	0.02 ± 0.01 a
alanine	0.40 ± 0.06 a	0.25 ± 0.03 a	0.26 ± 0.04 a
valine	0.00 ± 0.00 b	0.08 ± 0.03 a,b	0.10 ± 0.03 a
proline	2.83 ± 1.23 a	2.97 ± 0.97 a	2.52 ± 0.67 a
arginine	1.48 ± 0.40 a	1.57 ± 0.49 a	2.61 ± 0.55 a
isoleucine	0.03 ± 0.01 a	0.06 ± 0.01 a	0.05 ± 0.02 a
leucine	0.09 ± 0.02 b	0.16 ± 0.03 a	0.15 ± 0.02 a,b
tryptophan	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
phenylalanine	0.01 ± 0.01 a	0.00 ± 0.00 a	0.00 ± 0.00 a
cysteine	0.11 ± 0.06 b	0.32 ± 0.06 a	0.21 ± 0.06 a,b
ornithine	0.08 ± 0.03 a	0.00 ± 0.00 b	0.00 ± 0.00 b
lysine	0.30 ± 0.04 a	0.30 ± 0.05 a	0.36 ± 0.05 a
histidine	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
tyrosine	0.15 ± 0.07 a	0.21 ± 0.04 a	0.17 ± 0.06 a
total	8.31 ± 1.39a	7.93 ± 0.96 a	8.16 ± 1.06 a

[a] In the same row, between different collection times, means with different letters are significantly different ($p \leq 0.05$).

Table 3. Free Amino Acid Composition of Tronchuda Cabbage Internal Leaf Samples (Mean ± SD) (g/kg of Fresh Weight) at Different Collection Times[a]

amino acid (g/kg)	November 2005	December 2005	January 2006
aspartic acid	0.10 ± 0.05 b	0.42 ± 0.02 a	0.33 ± 0.06 a
glutamic acid	0.21 ± 0.04 a	0.28 ± 0.09 a	0.36 ± 0.10 a
asparagine	0.02 ± 0.02 a	0.08 ± 0.09 a	0.82 ± 0.72 a
glutamine	0.44 ± 0.13 a	0.69 ± 0.45 a	0.73 ± 0.24 a
serine	0.10 ± 0.03 a	0.47 ± 0.31 a	0.32 ± 0.27 a
threonine	0.40 ± 0.17 a	1.42 ± 0.43 a	1.26 ± 0.91 a
glycine	0.00 ± 0.00 a	0.07 ± 0.07 a	0.09 ± 0.04 a
alanine	0.26 ± 0.04 a	0.23 ± 0.09 a	0.41 ± 0.07 a
valine	0.02 ± 0.02 a	0.00 ± 0.00 a	0.00 ± 0.00 a
proline	0.19 ± 0.09 a	0.02 ± 0.02 a	0.03 ± 0.03 a
arginine	2.27 ± 0.42 b	3.61 ± 0.54 a,b	4.95 ± 0.67a
isoleucine	0.03 ± 0.02 a	0.03 ± 0.02 a	0.06 ± 0.03 a
leucine	0.02 ± 0.02a	0.07 ± 0.08 a	0.03 ± 0.02 a
tryptophan	0.01 ± 0.01 a	0.00 ± 0.00 a	0.05 ± 0.051 a
phenylalanine	0.04 ± 0.03 a	0.00 ± 0.00 a	0.01 ± 0.01 a
cysteine	0.24 ± 0.14 a	0.55 ± 0.21 a	0.48 ± 0.18 a
ornithine	0.08 ± 0.04 a	0.06 ± 0.04 a	0.07 ± 0.03 a
lysine	0.06 ± 0.03 a	0.07 ± 0.03 a	0.06 ± 0.03 a
histidine	0.08 ± 0.03 a	0.16 ± 0.09 a	0.07 ± 0.03 a
tyrosine	0.09 ± 0.03 a	0.06 ± 0.01 a	0.04 ± 0.02 a
total	4.64 ± 0.60 b	8.32 ± 1.02 a,b	10.16 ± 1.51 a

[a] In the same row, between different collection times, means with different letters are significantly different ($p \leq 0.05$).

obtained primarily from meat sources. Although, according to Sharma et al. (*11*), it accumulates in plants that are under heavy metal exposure. It seems that this amino acid can effectively protect plants from heavy metal attack. This protective effect may be explained by heavy metal detoxification through the formation of a nontoxic heavy metal−proline complex. *B. oleracea* var. *costata* leaves seem to be well-protected against heavy metals. The lowest heavy metal content that proline detoxification provides is beneficial to consumers' health.

Generally, threonine and lysine contents are limiting factors in the biological value of many proteins, mostly those from plant origins (*10*). In tronchuda cabbage, free amino acids seem to compensate for its possibly low biological value proteins. Additionally, threonine, an essential amino acid, helps to maintain the proper balance of protein in the body. It is important for the formation of collagen and elastin in the skin and aids in fighting fatty deposits in the liver, when combined with aspartic acid and methionine. Lysine is also an essential amino acid and is required for growth and bone development in children and assists in calcium absorption and in the maintenance of the correct nitrogen balance in the body, as well as lean body mass. Lysine is also needed to produce antibodies, hormones, enzymes, collagen formation, as well as to repair tissues.

Glutamine, a nonessential amino acid, is found in large amounts in the muscles of the body. Since glutamine passes easily through the blood−brain barrier, it is also known as an excellent brain fuel. This amino acid is converted to glutamic acid in the brain, which is essential for proper brain function, and increases the amount of gamma-amino butyric acid (GABA), which is required for brain function and mental activity. Glutamine is a source of fuel for cells lining the intestines, and it is also used by white blood cells, so it is important for immune function.

Although cysteine is a sulfur-containing nonessential amino acid, it can partly replace methionine (essential), which has the main role of being a methyl donor in many biochemical processes (to detoxify the body and its organs) (*10*). Cysteine is necessary in the detoxification of the body from harmful toxins, helping to protect the liver and brain from damage. It is required for the production of taurine and is a component of glutathione.

Glutamic acid (nonessential amino acid) is synthesized from a number of amino acids including ornithine and arginine, as it is important in the metabolism of sugars and fats. It also helps with the transportation of potassium across the blood−brain barrier (although glutamic acid itself does not pass this barrier that easily). Glutamate is part of the folate molecule, so that is why it is deemed a nonessential amino acid, as the body can usually get enough of it through adequate folate in the diet.

Aspartic and glutamic acids provide the umami taste or perception of satisfaction, which is an overall food flavor sensation induced or enhanced by monosodium glutamate (*12*). In humans, the taste receptor is far more sensitive to glutamate than to other amino acids (*13*). Other amino acids appear to exhibit sweet, bitter, or less intense tastes. Alanine, serine, glycine, and threonine are known to have sweet tastes, while leucine, phenylalanine, isoleucine, valine, histidine, arginine, and tryptophan present bitter flavors. Lysine, cysteine, and tyrosine are considered tasteless amino acids (*12*).

Several authors described the antioxidant effect of several amino acids in various matrices (*14–17*), and, for instance, tryptophan, cysteine, alanine, and glycine exert a synergistic effect with ascorbic acid on the antioxidant activity of vitamin E (*17*). All the referred amino acids were found in the analyzed samples and since tronchuda cabbage contains large amounts of free amino acids, it seems that they can confer or, at least, enhance the antioxidant capacity of this variety. In fact, the previously analyzed aqueous lyophilized extracts of *B. oleracea* var. *costata* leaves revealed a great antioxidant potential (*5, 6*).

As far as we know, this is the first time that the free amino acid composition is reported for tronchuda cabbage leaves, and this quantitative profile seems to be quite unusual. In 1996, Eppendorfer and Bille (*18*) determined the free and total amino acid composition of edible parts of beans, kale, spinach, cauliflower, and potatoes and found that free glutamine was dominant in kale and cauliflower. Latter, Gent (*19*) reported the free amino acid profile of seven salad greens, including

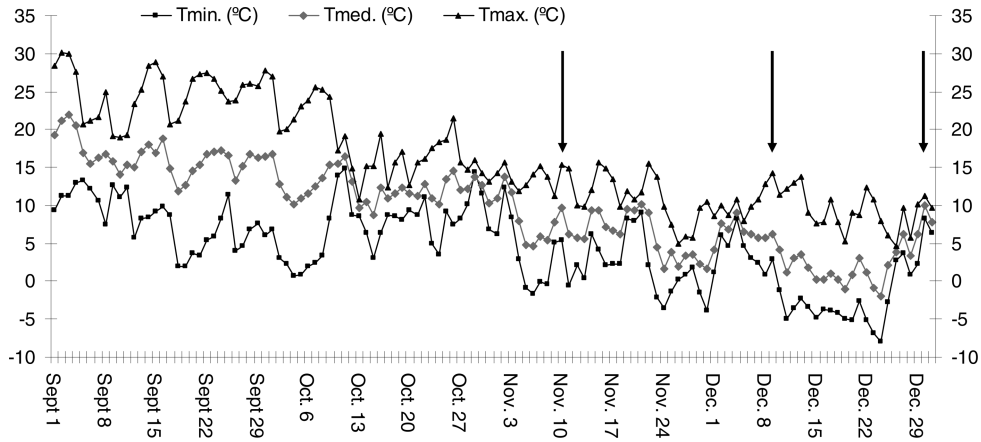

Figure 3. Environmental temperatures during the experiment. T_{min}: Daily minimum temperature; T_{med}: daily medium temperature; T_{max}: daily maximum temperature; and →: collection date.

leaves of kale (*B. oleracea*) cv. Red Russian, mibuna (*Brassica campestris*) cv. Green spray, and mustard (*Brassica juncea*) cv. Osaka purple. The sum of free amino acids ranged from 40 to 160 mmol/kg dry weight (or 4−16 g/kg dry weight, assuming an average molecular weight of 100), which is lower than that found in tronchuda cabbage leaves, and glutamic acid and/or glutamine were predominant amino acids in the leaves of all species (*19*). In 2006, Ayaz et al. (*20*) determined the amino acid profile of kale leaves (*B. oleracea* var. *acephala*). The most abundant amino acids (free plus bound) were glutamic and aspartic acids, arginine, leucine, proline, and valine (12.2, 10.2, 7.6, 7.5, 6.5, and 6.3% of the total amino acid content, respectively). The total (free plus bound) amino acid content was 271 g/kg dry weight. Gomes and Rosa (*4*) studied the free amino acid profile of primary and secondary inflorescences of 11 broccoli (*B. oleracea* var. *italica*) cultivars and its variation between seasons. These authors reported that the major amino acids were glutamine, glutamic and aspartic acids, alanine, and asparagine. That study indicated that free amino acids were not similarly distributed in the primary and secondary inflorescences since their content tends to be higher in the primary ones in the summer/winter season and in the secondary ones in the spring/summer. The total free amino acid content varied from 158 to 391 mmol/kg dry weight (or 16−39 g/kg dry weight, assuming an average molecular weight of 100) (*4*), which is lower than that found in tronchuda cabbage leaves.

As described by Gomes and Rosa (*4*), *B. oleracea* var. *costata* leaf free amino acids are not similarly distributed in internal (younger) and external (older) leaves. Proline and arginine were the major free amino acids, in external leaves, representing ∼56% of the total free amino acid content (31.9 and 24.2%, respectively). Threonine, glutamine, glutamic acid, and lysine were present in medium proportions (5−6% of total free amino acid content). However, internal leaves presented a different quantitative profile. Arginine was the main free amino acid (46.9% of total free amino acid content), followed by threonine, glutamine, and cysteine (11.9, 8.2, and 6.5% of total free amino acid content, respectively). Proline was present in a smaller amount (1.7% of total free amino acid content) than in external leaves. Significant differences were observed between leaf types for valine ($F = 9.469$; $d.f. = 1, 34$; $p = 0.004$), proline ($F = 30.249$; $d.f. = 1, 34$; $p \leq 0.001$), arginine ($F = 13.696$; $d.f. = 1, 34$; $p \leq 0.001$), leucine ($F = 12.454$; $d.f. = 1, 34$; $p = 0.001$), cysteine ($F = 4.191$; $d.f. = 1, 34$; $p = 0.048$), lysine ($F = 93.434$; $d.f. = 1, 34$; $p \leq 0.001$), histidine ($F = 12.591$; $d.f. = 1, 34$; $p = 0.001$), and tyrosine ($F = 12.341$; $d.f. = 1, 34$; $p = 0.001$) (**Figure 2**), although the total free amino acid content was similar in both kinds of leaves (mean values of 8.1 and 7.7 g/kg fresh weight for external and internal cabbage leaves, respectively).

No statistically differences were observed between the three different harvesting periods for the total free amino acid content in external leaves. However, marked differences were observed for glutamic acid, serine, and ornithine contents, which decreased significantly ($p \leq 0.05$) from November to January, and valine, leucine, and cysteine abundances, which increased significantly ($p \leq 0.05$) during this period (**Table 2**).

In internal leaves, the total free amino acid content increased significantly ($p \leq 0.05$) from November (4.6 g/kg fresh weight) to January (10.2 g/kg fresh weight). Concerning individual compounds, only aspartic acid and arginine showed significant variations (**Table 3**).

Gomes and Rosa (*4*) concluded that the levels of free amino acids of broccoli varieties were affected by climatic conditions, being reduced when the plant was submitted to stress (*4*). However, according to our results, no differences were observed in tronchuda cabbage external leaves (**Table 2**), and significant increases were registered in internal ones (**Table 3**), even though extreme temperatures (less than zero) were observed during various days in December (**Figure 3**). This fact can most likely be related to genetic factors of this cabbage species. In plant species that are not adapted to low temperatures (below 0 °C), the crystallization of water promotes the rupture of membrane cells and the destruction of plant tissues. Tronchuda cabbage seems to be well-adapted, and its tissues are maintained in good condition for several days at low temperatures (*8*). Generally, in the north of Portugal, tronchuda cabbage is cultivated in the autumn/winter.

Free amino acids were identified as the precursors of several classes of secondary plant metabolites, namely, phenolic acids, flavonoids, and glucosinolates, although, as far as we know, there are few reports concerning the relationship between those secondary metabolites and their precursors in vivo. Considering that methionine, phenylalanine, tyrosine, and tryptophan are precursors of glucosinolates, Rosa and Gomes (*21*) investigated the relationship between these two classes of compounds in 11 broccolli cultivars grown early (April−July) and late (August−January). They concluded that in this matrix, there is no correlation between the glucosinolates and their precursors, the free amino acids.

This study attempted to contribute to the knowledge of nutritional properties of an important Portuguese crop. In conclusion, the two kinds of tronchuda leaves (external and internal) in a diet supply different proportions of free amino acids. It is important to consume both kind of leaves to obtain

the appropriate amounts of amino acids and a great diversity of palatable sensations. In addition, these cabbages are of great nutritional importance because they are rich in free amino acids (mean value of 7.9 g/kg fresh weight), which are indispensable in a healthy diet.

In external leaves, proline and arginine are the major free amino acids, while in internal ones, arginine is the main free amino acid, followed by threonine, glutamine, and cysteine. Significant differences between leaf types were observed in the quantitative profile, namely, in the valine, proline, arginine, leucine, cysteine, lysine, histidine, and tyrosine contents. Significant differences among harvesting periods also were observed in the free amino acid profiles (glutamic acid, serine, valine, leucine, cysteine, and ornithine contents and aspartic acid, arginine, and total contents in external and internal leaves, respectively).

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4.10. Volatile composition of *Brassica oleracea* L. var. *costata* DC leaves using solid phase microextraction and gas chromatography/ion trap mass spectrometry

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Volatile composition of *Brassica oleracea* L. var. *costata* DC leaves using solid phase microextraction and gas chromatography/ion trap mass spectrometry

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Running Tittle : Volatile profile of *Brassica olearacea* L. var. costata DC leaves

ABSTRACT

Volatile and semi-volatile components of internal and external leaves of *Brassica oleracea* L. var. *costata* DC, grown under different fertilization regimens, were determined by headspace solid-phase microextraction (HS-SPME) combined with gas chromatography / Ion Trap-Mass Spectrometry (GC/IT-MS). Forty one volatiles and non-volatiles components were formally identified and thirty others were tentatively identified. Qualitative and quantitative differences were noticed between internal and external leaves. In general, internal leaves exhibited more aldehydes and sulfur volatile compounds than external ones, and less ketone, terpenes and norisoprenoids compounds. The fertilization regimens influenced considerably the volatile profile. Fertilizations with higher levels of sulfur produced *Brassica* leaves with more sulfur volatiles. In opposition, N and S fertilization lead to leaves with lower levels of norisoprenoids and terpens.

KEYWORDS: *Brassica oleracea* L. var. *costata* DC; Volatile, semi-volatile compounds; GC-IT-MS; Solid Phase MicroExtraction.

INTRODUCTION

Plants from the Brassicaceae family play a major role in worldwide vegetable production and consumption, ranking second after the Solanaceae. *Brassica* vegetables, including all cabbage like ones are consumed in great quantities all over the world. *Brassica oleracea* L. var. *costata* DC (tronchuda cabbage) is still considered to be a primitive cultivar being high yielding, less susceptible to pests and diseases, well adapted to a wide range of climates, and generally grown with little or no agrochemical input [1,2]. *Brassica* species are reported to have anti-cancer activity [3,4]. Within *Brassica* genus, *B. oleracea* species have a huge number of varieties of which different parts of the plant have become edible [5].

In spite of all the attention focused on *Brassica* vegetables, the characterization of natural products other than glucosinolates[6,7] in this plant remains scarce. Recently some works were performed concerning their phenolics and organic acids profiles[8-10]. Several studies have reported the volatile composition of different species of *Brassica*, namely *Brassica rapa L. var. perviridis* Bailey[11] *and Brassica olearacea L. var. botrytis L.* [12]. These two previous works used hydrodistillation and organic solvents to extract volatiles and had screening this kind of compounds in the leaves. These works permitted the identification of several types of compounds, namely alkanes, aldehydes, ketones, fatty acids and their esters, terpenoids, alcohols and some sulfur compounds [11,12]. These two extraction methodologies have traditionally been applied for essential oil extraction from plant material[13-15], although they present some shortcomings, such as losses of volatile compounds, low extraction efficiency and long extraction time. Also, high temperatures and water can cause degradation or chemical modifications of volatile constituents[16].

In recent years, the most frequent analytical techniques applied in the extraction and concentration of volatile compounds from plants, fruits, beers, wines are those based on headspace analysis (HS) [17-21]. Among the headspace methods, the Solid-Phase MicroExtraction (SPME) constitutes a reliable tool for the analysis of organic volatile and also semi-volatile compounds. SPME is a technique that shows clear advantages over other traditional ones: high sensibility and reproducibility; low cost; relative simplicity. Unlike other conventional methods which require extensive sample preparation, SPME is a one step extraction procedure in which the compounds of interest are absorbed by a thin polymer film or by a porous carbonaceous material bonded to a fused silica fiber. HS-SPME technique combined with ion trap mass

spectrometry (ITMS) is capable of producing full scan mass spectra at very low concentration levels.

The aim of this work was to extend the knowledge of volatile compounds of *Brassica oleracea* L. var. *costata* DC using the HS-SPME technique directly into the headspace of leaves (internal and external). The application of this extraction technique, allied to GC-ITMS, constitutes an interesting and novel detailed analysis of volatile compounds of this species, additionally allowing the discrimination of the distinct leaves and the influence of fertilization treatments.

MATERIALS AND METHODS

Standards. Reference compounds were purchased from various suppliers: 2-methylbutanal, pentanal, hexanal, *(E)*-2-hexenal, octanal, *(E)*-2-octenal, *(E,E)*-2,4-nonadienal, *(E)*-2-nonenal, *(E,E)*-2,4-decadienal, *(E,Z)*-2,6-nonadienal, *(E)*-2-nonenol, 2,2,6-trimethylcyclohexanone, geranylacetone, oleic acid, benzenepropanenitrile, *β*-cyclocitral, *β*-homocyclocitral, eugenol, ethyl linoleanate, hexanoic acid methyl ester, octanoic acid ethyl ester, decanoic acid ethyl ester, hexadecanoic acid ethyl ester, 6-methyl-5-hepten-2-one, □-pinene, and limonene were from Sigma (St. Louis, MO, USA); benzaldehyde, (*E*)-2-decenal, 1-octen-3-one, β-ionone, dimethyl disulfide, dimethyl trisulfide, *n*-butyl isothiocyanate, hexylisothiocyanate, 3-methylthiopropylisothiocyanate and isothiocyanic acid phenethylester were obtained from SAFC (Steinheim, Germany); *o*-cimene, eucalyptol, were from Extrasynthese (Genay, France); and menthone and *(E,E)*-farnesylacetone from Fluka (Buchs, Switzerland). allylisothiocyanate was from Riedel de Haën (Seelze, Germany).

Plant Material and Treatments. Tronchuda cabbage (*B. oleracea* var. *costata*) plants were grown under different fertilization regimes. The experimental work was carried out in one field located in Bragança, northeastern Portugal (41° 48′ N, 6° 44′ W). The field had an inclination inferior to 5% and was turned up to the northeast. Sowing occurred in the middle of June 2005, in a greenhouse (22 °C, 80% humidity).

Young plants were transplanted to the field at the end of August, spaced at 0.8 × 0.5 m between and within rows. Before the fertilization treatments, the soil was loamy textured with 0.83% organic matter, a pH (H2O) of 5.2, and median phosphorus (54 mg of P2O5/kg) and high potassium (126 mg of K2O/kg) levels. Four treatments were established: a control (C), without any fertilization, and one with Dix10 (Crimolara, Portugal), an authorized organic amendment (10% total N, 3% K2O, 3% P2O5, 2.5% CaO, 0.6% MgO, and 30.5 mg of B/kg), and finally S1 and S2, with 37.3 and 74.6 kg of S/ha, respectively. All conventional fertilizer regimens received 80 kg of N/ha to ensure plant growth. The fertilizers were simultaneously applied at the beginning of the growth season. The conventional fertilizer used was magnesium sulphate (ADP, Portugal). Phosphorus (150 kg of super 18/ha) and potassium (50 kg of KCl/ha) were also used in these fields. Twelve cabbages were planted for each experimental treatment (**Table 1**).

Samples were collected in mid-November 2005. For each fertilization regimen three plants was randomly collected from three different plots. All samples were collected in the morning, at the same hour. After harvesting, the plants were immediately transported to the laboratory and weighed, and external and internal leaves were separated. Care was taken to choose plants and leaves of similar developmental stage: internal leaves, looking pale yellow and tender, were separated from the external ones, which presented a dark green colour and were no longer actively expanding, although not yet senescent.

Each analyzed sample corresponds to the mixture of the three plants developed and collected in the same conditions. Two hours maximum after their collection, the samples were frozen at -20 °C and then lyophilized (Labconco 4.5 Freezone apparatus, Kansas City, MO). The freeze-dried samples were powdered and kept in a desiccator in the dark.

SPME technique. *SPME fibres*. Several commercial fibres can be used to extract volatiles. According to bibliography[17, 20, 21], recommendations of supplier (Supelco, Bellefonte, PA, USA) and to our own knowledge based on experimental work, the fibre coated with Divinylbenzene/PDMS (DVB/PDMS) as stationary phases and 65 µm film thickness is the most adaptable to the intended compounds and to the matrix under study. It was conditioned by inserting into the GC injector; temperature and time were used according to the procedure recommendation of Supelco: 250 ºC for 30 minutes.

Headspace Solid Phase Microextraction (HS-SPME) – powder. Approximately 25 mg of freeze-dried powered leaves were directly submitted to the SPME technique. Samples were stirred at 600 rpm, at 40 ºC for 5 min. The fibre was then exposed to the headspace for 20 min, with agitation (800 rpm), according to the methodology described before[20, 21]. Afterwards the fibre was pulled into the needle sheath and the SPME device was removed from the vial and inserted into the injection port of the GC system for thermal desorption**.** After 1.5 min the fibre was removed and conditioned in another GC injection port for 15 min at 250 ºC.

Headspace Solid Phase Microextraction (HS-SPME) – solution. Approximately 0.1 g of freeze-dried powered samples was dissolved in 5 mL of a 5% ethanol solution in a 15 mL vial, and 0.5 g of anhydrous sodium sulphate was added to favour the release of analytes from the matrix. It was then sealed with a polypropylene hole cap and PTFE/silicone septa (Supelco, Bellefonte, PA, USA). The mixture was

then magnetically stirred at 760 rpm, at 40 ºC, for 5 min. The fibre was then exposed to the headspace for 20 min, with agitation (800 rpm). Afterwards, the fibre was pulled into the needle sheath and the SPME device was removed from the vial and inserted into the injection port of the GC system for thermal desorption**.** After 1.5 min the fibre was removed and conditioned in another GC injection port for 15 min at 250 ºC. This methodology is adapted from previous work performed in wine matrix[17]. All samples were analysed in triplicate.

Gas Chromatography-Mass Spectrometry analysis. HS-SPME analysis was performed using a Varian CP-3800 gas chromatograph (USA) equipped with a VARIAN Saturn 4000 mass selective detector (USA) and a Saturn GC/MS workstation software version 6.8. The column used for samples analysis was VF-5ms (30 m x 0.25 mm x 0.25 μm) from VARIAN. Stabilwax-DA fused silica column (60 m x 0.25 mm, 0.25 μm) (Restek, USA) was used in order to check the identity of some compounds found in the first column. The injector port was heated to 220 °C. The injections were performed in a splitless mode. The carrier gas was Helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min. The oven temperature was set at 40 °C for 1 min, then increasing 2 °C/min to 220 °C and held for 30 min. All mass spectra were acquired in the electron impact (EI) mode. Ionization was maintained off during the first minute. The Ion Trap detector was set as follows: the transfer line, manifold and trap temperatures were respectively 280, 50 and 180 ºC. The mass ranged from 40 to 300 m/*z*, with a scan rate of 6 scan/s. The emission current was 50 μA, and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionization time was 25000 μs, with an ionization storage level of 35 m/z.

Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic compounds analysed under the same conditions, and by comparison of the retention indices (as Kovats indices) with the literature data. The comparison of MS fragmentation pattern with those of pure compounds and mass spectrum database search was performed using the National Institute of Standards and Technology (NIST) MS 05 spectral data base. Confirmation was also conducted using the laboratory built MS spectral database, collected from chromatographic runs of pure compounds performed with the same equipment and conditions. The relative amounts (RAs) of individual components are expressed as percent peak areas relative to total peak area. Chromatographic peaks' areas were determined by re-constructed FullScan chromatogram using for each compound some

specific ions, quantification ions (see Table 1). By this way some peaks which are co-eluted in FullScan mode (Resolution value less than 1) can be integrated with a value of Resolution higher than 1.

Statistical analysis. Principal component analysis (PCA) was carried out using XLSTAT 2007.5 software. PCA method shows similarities between samples projected on a plane and makes it possible to identify which variables determine these similarities and in what way. Analysis of variance (ANOVA) using Excel ™ software from Windows 98 v 7.0 was applied to the experimental data, the results were considered significant if the associated p-value was < 0.05.

Table 1. Characterization of Brassica samples

Levels of treatment	Specifications
S0 int	Control: Internal leaves without fertilization
So ext	Control: External leaves without fertilization
S1 int	Internal leaves with 37.3 kg. of S/ha
S1 ext	External leaves with 37.3 kg. of S/ha
S2 int	Internal leaves with 74.6 kg. of S/ha
S2 ext	External leaves with 74.6 kg. of S/ha
Dix10 int	Internal leaves with organic amendment
Dix10 ext	External leaves with organic amendment

RESULTS AND DISCUSSION

Analytical conditions. The Divinylbenzene/PDMS fiber was selected for the analysis of all samples, as it revealed to be the best and more selective fiber for the identification of aldehydes, sulfur and nitrogen volatile compounds[21], considered as important to the cabbage characterization[11,12]. HS-SPME analysis was performed both in the lyophilized cabbage leaves and in the lyophilized cabbage leaves mixed with a 5% ethanol solution. The last one was selected as it was demonstrated, by previous works, that the better equilibrium liquid/gas for the majority of volatile compounds is performed in a solution of 5 % ethanol [20]. By this way less water soluble compounds can also be liberated to gas phase. A large range of polarity compounds is taken, namely the isothiocyanates. For quantification analysis this procedure was used.

Aroma composition. Solid phase microextraction allowed the identification of 71 volatile compounds in the analyzed *Brassica* leaves. These include a total of 1 acetal (**1**), 1 volatile acid (**2**), 17 aldehydes (**3-19**), 2 alcohols (**20**, **21**), 7 methyl and ethyl esters (**22-28**), 7 ketones (**29-35**), 16 volatile sulfur compounds (**36-51**), 8 terpenes (**52-59**), 8 norisoprenoid compounds (**60-67**) and 4 other volatile compounds (**68-71**) (**Table 2 and Fig. 1**). Although, some peaks obtained by FullScan mode have not enough resolution to be used for quantification analysis, the re-constructed chromatogram using the specific m/z ions for each co-eluted peak, allow its quantification. An example is shown in **Fig. 2a**, where two compounds **20** and **48** with a difference of retention time of 0.046 minutes can be separated and quantified by using their specific m/z ions (**Fig 2b**).

In order to assess the influence of the leave position (internal and external leaves) and all treatments on the identified volatile compounds, a Principal Component Analysis (PCA) was performed. **Fig. 3** shows the projection of all chemical variables, grouped by families, into the plans F1 and F2, corresponding to 68.75% of the total variance. **Fig. 4** shows the projection of all samples in the two principal components, being the second principal component responsible for the separation of samples into two groups: one composed by the internal leaves (class 1), projected in the left side, and the second by the external leaves (class 2), on the right side. The variables which are responsible for this classification are: "Sulfur", corresponding to the total sulfur compounds, and "Aldehydes" (**Table 2 and Fig. 3**). In fact, according to this analysis, the external leaves, independently of the fertilization treatment, are richer in norisoprenoid volatile compounds. In opposition, the internal leaves are richer in

aldehydes and sulfur compounds. The other group of volatiles, namely, alcohols, acids, esters, terpenes and miscellaneous compounds, has little influence on the differentiation of samples. A sequential PCA was performed in order to extract more information about samples. Thus, aldehyde and sulfur compounds were taken off from this novel PCA analysis. It can be observed that the same sample groups were formed (internal and external leaves groups) but the difference is that external leaves are richer in all these compounds when comparing to internal ones (**Figs. 5 and 6**). In fact, using all chemical families we are not able to differentiate samples according to the fertilization procedures. Hence, in order to try to understand the influence of each fertilization treatment on the volatile profile of *Brassica* leaves, an analysis of each chemical family was performed. In what concerns aldehydes (**Table 2**), there is a great variability among data, being the treatment with Dix10 responsible for the lowest levels of aldehydes in the respective leaves. Differences between treatments can be observed (**Table 2**).

Acetals, acids, alcohols and methyl and ethyl esters (**Table 2**) have a lower contribution to the total aroma composition of *Brassica* leaves. In addition, ketones represent between 10 and 15% of the total volatiles. This contribution is mainly due to 3,5-octadien-3-one and *(E,E)*-3,5-octadiene-3-one levels (**Table 2**). In tropical fruits, the presence of these compounds confers sweet aromas[22]. In fact, previous works have pointed to a high sweet character of internal *Brassica* leaves[10]. The internal and external leaves were considerably different in what concerns to organoleptic characteristics, which may influence the preferences of consumers[10].

Among the identified ketones, another group, composed by volatile norisoprenoids like α and β-ionone, epoxy-β-ionone, emerged (**Table 2**). Levels of norisoprenoids can attaint 50% of the total volatiles in some samples. These odor-active substances are known to be oxidative by-product or degradation products derived from carotenoids[22,23]. They contribute to floral aromas as "violet like". The β-ionone is an important flavor in some wine varieties[25]. These compounds have never been identified in *Brassica*. The external leaves have much higher amounts of norisoprenoids, with exception of the internal leaves receiving Dix10 treatment (**Table 2**). Samples corresponding to this treatment have higher amounts in 1,3,4-trimethyl-3-cyclohexen-1-carboxaldehyde, β-cyclocitral and β-ionone. It seems that the Dix 10 treatment induced the biosynthesis of carotenoid molecules, the ANOVA treatments of the data showed differences between treatments and between the different compounds $p = 0.001249$ and $p = 3.33e^{-05}$, respectively, at the 95% level.

In spite of the lower contribution of terpene molecules to the total amount of volatiles, differences between fertilizations and external and internal leaves can be also observed in this family **(Table 2).** Volatile terpenes exist in higher amounts in external leaves, compared with internal ones, which is in accordance with the fact that terpenoid molecules have an important role in tritrophic insect-plant interactions[26].

Sulfur compounds, such as disulfides, trisulfides and tetrasulfides, and isothiocyanates were identified in all samples. Isothiocyanates are derived from the hydrolysis of glucothiocyanates in presence of myrosinase and water. Recent works[27] show the influence of nitrogen and sulfur fertilizers on the concentration of carotenoids and glucosinolates in watercress. Glucosinolates are one of the most important groups of molecules presents in *Brassica*. Several works show their importance in human health[28,29]. For example, 2-phenethyl isothiocyanate has received attention for its role in the reduction of carcinogen activation through inhibition of phase I enzymes (such cytochrome P450s) and its potential to induce phase II enzymes[30]. The isothiocyanates play also an important role in aroma of these vegetables, mainly when they are processed[31]. According to previous studies[32,33] the *Brassicaceae* (cabbage, broccoli, mustard and others) aroma can be correlated with tetrahydrothiophene, allyl isothiocyante, 4-methylthiobutyl pentanenitrile 4-pentenyl isothiocyanate, 4-methylthiobutyl isothiocyanate, 5-methylthio penthyl isothiocyanate[32], being allyl isothiocyanate and 4-pentenyl isothiocyanate responsible for the horseradish aroma[33]. As it can be observed in **Table 2** these kinds of compounds exist in *Brassica* leaves in high percentage. Levels are higher in S1 and S2 samples (which correspond to the treatment with sulfur fertilizer) and in both cases in the internal leaves. These results are in accordance with those obtained for glucosinolates[27]. Disulfides, trisulfides and tetrasulfides are also detected and their presence confers the "cabbage" aroma to *Brassica*. It seems that sulfur fertilizers induced the production of isothiocyanates, which is an advantage for human health; nevertheless, the concomitant production of sulfides can be taken into account in what concern to consumer preferences.

The employment of HS-SPME techniques combined with GC-ITMS permitted the identification and quantification of a large number of compounds in all studied samples. In addition, it was possible to discriminate perfectly the internal and external leaves in two distinct groups. The influence of fertilization is more difficult to assess; nevertheless, the amendment with higher levels of sulfur fertilizations induced the production of higher amounts of sulfur and aldehyde volatile compounds.

This work is the first approach to the volatile characterization of *Brassica oleracea* L. var. *costata* DC concerning the fertilization treatments. Further works concerning the influence of some edafo-climatic conditions, such as soil characteristics, sunlight and shade exposure, altitude of plantation, irrigation, could be also performed as they have certainly influence in the final volatile composition of Brassica vegetables. The volatile profile of Brassica vegetables during its development stages and the time and temperature of storage would be also interesting to study.

Table 2. Relative percentage (%) of the compounds identified in *Brassica oleracea* L. var. *costata* DC using HS-SPME.

Number	Compound	Retention Index	Quantification ions (m/z)	ID[a] (Fit/Rfit)	RA[b](%) S0ext	RA(%) S0int	RA(%) S1ext	RA(%) S1int	RA(%) S2ext	RA(%) S2int	RA(%) Dix10ext	RA(%) Dix10int
	Acetals											
1	Diethylacetal	834	45/73/103	MS(83.6/84.0)[c]	12,469	0,301	0,318	2,340	10,821	8,439	8,491	14,673
	Acids											
2	Oleic acid	2142	55/97/157/256	S[d],MS	0,242	0,175	0,227	0,247	0,187	0,433	0,272	0,471
	Aldehydes											
3	2-Methylbutanal	771	41/57/58	S,MS	nd	nd	nd	0,525	nd	0,022	nd	nd
4	Pentanal	803	44/58/81	S,MS	0,307	0,218	0,532	0,422	0,271	0,335	0,213	0,368
5	(*E*)-2-Pentanal	866	55/83	MS(70.3/75.2)	0,062	0,093	nd	0,117	0,020	0,059	0,033	0,058
6	Hexanal	910	56/67/83/99	S,MS	0,796	0,259	0,426	0,666	0,358	0,365	0,185	0,320
7	(*E*)-2-Hexenal	966	55/69/83/99	S,MS	0,344	0,170	0,343	0,229	0,113	0,108	0,237	0,409
8	(*Z*)-4-Heptenal	1008	67/84	MS(70.1/84.8)	0,179	0,026	0,248	0,028	0,250	nd	0,147	0,254
9	(*Z*)-2-Heptenal	1069	55/69/83	MS(76.9/87.6)	0,245	3,969	1,662	4,921	nd	3,551	0,399	0,690
10	Benzaldehyde	1076	77/105	S,MS	1,141	0,253	0,845	0,698	1,000	0,254	0,066	0,114
11	(*E*,*E*)-2,4-Nonadienal	1097	81	S,MS	0,835	0,330	nd	0,397	0,487	0,306	0,217	0,375
12	(*E*,*E*)-2,4-Heptadienal	1108	53/81	MS(80.2/81.5)	2,400	8,708	6,252	7,258	3,313	6,405	1,791	3,095
13	Octanal	1113	67/81/95	S,MS	0,287	0,257	6,072	0,451	3,274	0,218	0,218	0,376
14	(*E*)-2-Octenal	1171	55/70/83	S,MS	nd	3,480	1,294	4,817	nd	nd	nd	nd
15	(*E*,*Z*)-2,6-Nonadienal	1265	70/95/137/152	S,MS	0,355	1,488	1,470	1,201	0,761	0,680	0,306	0,530
16	(*E*)-2-Nonenal	1272	55/70/83	S,MS	0,090	0,684	0,254	0,336	0,120	0,414	nd	nd
17	(*E*,*E*)-2,4-Decadienal	1316	81	S,MS	0,221	1,117	nd	1,272	nd	0,353	nd	nd
18	(*E*)-2-Decenal	1341	55/70/83/97	S,MS	0,161	nd	0,563	2,685	1,025	0,845	0,311	0,537
19	10-Undecenal	1390	55/83/95/121	MS(81.1/82.9)	0,048	0,945	0,720	2,260	0,077	0,609	nd	nd
			Σ		7,471	21,997	20,681	28,283	11,069	14,524	4,123	7,126
	Alcohols											
20	2-Nonenol	1215	57/81/95	S,MS	1,710	1,603	2,266	2,200	4,827	1,541	1,602	2,769
21	*trans*-2-Decenol	1310	67/81/95/109	MS(81.9/84.9)	0,984	1,473	1,046	nd	1,397	0,950	0,887	1,532
			Σ		2,694	3,076	3,312	2,200	6,224	2,491	2,489	4,301
	Esters											
22	Pentanoic acid ethyl ester	1009	88/101	MS(81.7/85.1)	nd	0,184	nd	0,201	nd	0,210	0,077	0,133
23	Hexanoic acid methyl ester	1035	74/87/99	S,MS	0,099	0,093	0,218	0,369	0,144	0,057	0,101	0,174
24	Octanoic acid ethyl ester	1302	88/140	S,MS	0,879	1,000	1,848	1,272	0,676	1,343	0,691	1,193
25	Decanoic acid ethyl ester	1404	88/157	S,MS	0,275	0,306	0,803	0,092	nd	0,526	0,453	0,783
26	Undecanoic acid ethyl ester	1504	88/157	MS(78.6/79.1)	0,058	0,077	nd	nd	nd	0,099	0,088	0,153
27	Hexadecanoic acid ethyl ester	1905	88/157/284	S,MS	nd	nd	nd	nd	nd	0,724	nd	nd
28	Ethyl linoleanate	1978	79	S,MS	0,972	nd	1,169	0,135	0,696	0,366	nd	nd
			Σ		2,284	1,661	4,037	2,068	1,516	3,325	1,409	2,436

[a]ID, identification method (Fit/Retrofit values-%), Retention Index – determined in a VF-5ms (30 m x 0.25 mm x 0.25 µm) from VARIAN
[b]RA(%), relative area in percentage
[c]MS, tentatively identified by NIST 05
[d]S, identified by comparison with reference compound
nd[e], not detected

Table 2. Relative percentage (%) of the compounds identified in *Brassica oleracea* L. var. *costata* DC using HS-SPME (Continuation).

	Compound	Retentin Index	Quantification ions	ID[a] (Fit/Rfit)	RA[b](%) S0ext	RA(%) S0int	RA(%) S1ext	RA(%) S1int	RA(%) S2ext	RA(%) S2int	RA(%) Dix10ext	RA(%) Dix10int
	Ketones											
29	6-Methyl-2-heptanone	1065	91	MS[c](71.2/73.4)	0,044	nd	nd	nd	0,085	nd	nd	nd
30	1-Octen-3-one	1087	55/70/97	S[d],MS	nd	8,835	nd	12,647	nd	6,981	0,408	0,705
31	6-Methyl-5-hepten-2-one	1094	67/108	S,MS	0,785	0,154	0,731	0,054	0,679	nd	0,843	1,457
32	2,2,6-Trimethylcyclohexanone	1148	82/140	S,MS	1,336	0,222	2,633	0,176	1,837	0,254	0,985	1,702
33	Isophoron	1172	82/110	MS(81.4/82.9)	1,354	0,094	2,274	nd	1,530	0,091	1,266	2,189
34	*(E,E)*-3,5-Octadien-2-one	1180	81/95	MS(82,2/83,7)	9,505	0,770	4,389	0,329	7,166	0,804	3,859	6,669
35	(*E*,*E*)-farnesylcetone	1856	95/109/124	S,MS	0,441	0,422	0,698	0,178	0,290	0,312	0,634	1,096
			Σ		13,464	10,496	10,724	13,385	10,587	8,442	7,996	13,828
	Sulfur compounds											
36	Dimethyl disulfide	854	45/79/94	S,MS	0,354	2,970	0,469	5,503	1,839	1,775	0,181	0,314
37	Allyl isothiocyanate	984	41/72/99	S,MS	4,526	32,236	16,545	0,196	3,010	18,168	26,515	45,822
38	Butyl isothiocyanate	1065	57/72/115	S,MS	nd	0,251	nd	nd	nd	0,318	0,343	0,593
39	Dimethyltrisulfide	1082	45/79/126	S,MS	1,195	3,746	0,832	21,804	5,787	5,344	0,866	1,497
40	3-Butenyl isothiocyanate	1091	55/72/113	MS(76.1/80.1)	nd	1,087	0,537	nd	0,123	3,867	0,287	0,495
41	2-Methylbutyl Isothiocyanate	1169	71/100/129	MS(72.4/75.1)	nd	2,016	1,165	nd	nd	4,053	1,959	3,385
42	Pentyl isothiocyanate	1205	72/101/129	MS(70.7/75.4)	nd	nd	nd	nd	nd	0,507	0,140	0,242
43	4-Methylpentyl isothiocyanate	1272	72/110/128/143	MS(72.2/75.6)	nd	0,094	0,452	nd	nd	0,437	nd	nd
44	Hexyl isothiocyanate	1277	72/115/128	S,MS	nd	0,103	0,486	0,450	nd	0,487	2,056	3,553
45	Heptyl isothiocyanate	1304	72/115	MS(72.5/74.9)	nd	0,837	nd	1,134	nd	0,133	0,057	0,099
46	Dimethyltetrasulfide	1318	79/158	MS(82.5/86.6)	nd	0,431	8,202	13,589	nd	0,257	nd	nd
47	3-methylthiopropyl isothiocyanate	1366	61/72/101	S,MS	nd	nd	nd	nd	nd	nd	nd	nd
48	Phenylmethylthiocyanate	1390	91	MS(85.8/88.1)	nd	nd	nd	nd	0,707	nd	2,671	4,616
49	Octyl isothiocyanate	1417	72/115/138/170	MS(78.1/81.7)	nd	nd	0,230	nd	0,190	0,110	0,205	0,355
50	Isothiocyanic acid phenethylester	1480	91/105/163	S,MS	0,156	8,824	1,339	nd	0,267	14,647	0,634	1,095
51	Dimethylpentasulfide	1482	79/126/158	MS(76.2/83.9)	nd	nd	nd	0,339	nd	nd	nd	nd
			Σ		6,231	52,594	30,258	43,016	11,925	50,104	35,915	62,065

[a]ID, identification method (Fit/Retrofit values-%), Retention Index – determined in a VF-5ms (30 m x 0.25 mm x 0.25 µm) from VARIAN
[b]RA(%), relative area in percentage
[c]MS, tentatively identified by NIST 05
[d]S, identified by comparison with reference compound
nd[e], not detected

Table 2. Relative percentage (%) of the compounds identified in *Brassica oleracea* L. var. *costata* DC using HS-SPME (Continuation).

Number	Compound	Retention Index	Quantification ions	ID[a] (Fit/Rfit)	RA[b](%) S0ext	RA(%) S0int	RA(%) S1ext	RA(%) S1int	RA(%) S2ext	RA(%) S2int	RA(%) Dix10ext	RA(%) Dix10int
	Terpenic compounds											
52	*o*-Cimene	1045	93/121	S[d],MS[c]	0,234	0,183	nd	0,074	0,133	0,072	nd	nd
53	β-Pinene	1119	93/121	S,MS	0,555	0,429	0,219	0,161	nd	0,167	0,039	0,068
54	γ-Terpinene	1127	77/93/121/136	MS(81.2/89.1)	0,256	nd	0,189	nd	0,335	0,062	0,172	0,297
55	*m*-Cymene	1136	91/119	MS(81.2/82.4)	0,474	0,575	0,124	0,236	0,605	0,415	0,226	0,391
56	Limonene	1140	67/93	S,MS	0,853	0,533	0,527	0,347	0,885	0,421	nd	nd
57	Eucalyptol	1145	81/93/139	S,MS	0,051	nd	nd	nd	0,893	0,141	1,057	1,827
58	Menthone	1270	69/112/139	S,MS	nd	0,058	0,273	0,072	nd	0,070	0,408	0,705
59	α-Bisabolol	1701	93/119	MS(74.8/89.3)	0,013	nd	nd	nd	0,271	0,072	0,061	0,106
			Σ		2,435	1,779	1,331	0,891	3,122	1,421	1,964	3,393
	Norisoprenoids derivatives											
60	1,3,4-Trimethyl-3-cyclohexen-1-carboxaldehyde	1319	67/81/109	MS(82.4/83.3)	10,315	0,078	nd	1,134	nd	1,723	10,038	17,348
61	β-Cyclocitral	1319	109/137/152	S,MS	5,797	0,311	nd	0,857	11,509	1,426	8,808	15,221
62	β-Homocyclocitral	1339	107/151	S,MS	0,343	nd	0,325	0,214	0,358	0,327	0,251	0,434
63	α-Ionone	1437	93/121	MS(78.2/82.5)	0,554	0,490	0,537	0,284	0,388	0,244	0,289	0,499
64	Geranylacetone	1459	69/107/136	S,MS	3,007	0,227	1,295	0,106	1,694	0,142	2,360	4,078
65	β-Ionone	1493	177	S,MS	17,337	3,398	15,264	2,165	17,430	2,568	9,979	17,244
66	Epoxy-β-ionone	1496	123/177	MS(86.8/87.4)	7,085	0,465	3,042	0,089	2,943	0,285	1,257	2,173
67	Dihydroactinolide	1548	111/137/180	MS(83.5/88.8)	2,224	0,447	3,712	0,280	2,240	0,534	1,296	2,239
			Σ		46,662	5,417	24,174	5,130	36,563	7,249	34,278	59,236
	Miscellaneous compounds											
68	Toluene	876	91	MS(90.2/91.6)	0,519	0,284	0,529	0,295	2,325	0,414	0,357	0,616
69	4-Ethyl-phenol	1027	107/122	MS(75.5/85.3)	0,151	0,413	nd	0,242	0,088	0,275	0,077	0,134
70	Benzenepropanenitrile	1329	91/131	S,MS	nd	1,423	nd	1,672	0,391	0,275	0,283	0,489
71	Eugenol	1385	131/164	S,MS	nd	nd	nd	nd	0,016	0,032	0,013	0,022
			Σ		0,670	2,120	0,529	2,209	2,821	0,995	0,730	1,261

[a]ID, identification method (Fit/Retrofit values-%), Retention Index – determined in a VF-5ms (30 m x 0.25 mm x 0.25 µm) from VARIAN
[b]RA(%), relative area in percentage
[c]MS, tentatively identified by NIST 05
[d]S, identified by comparison with reference compound
nd[e], not detected

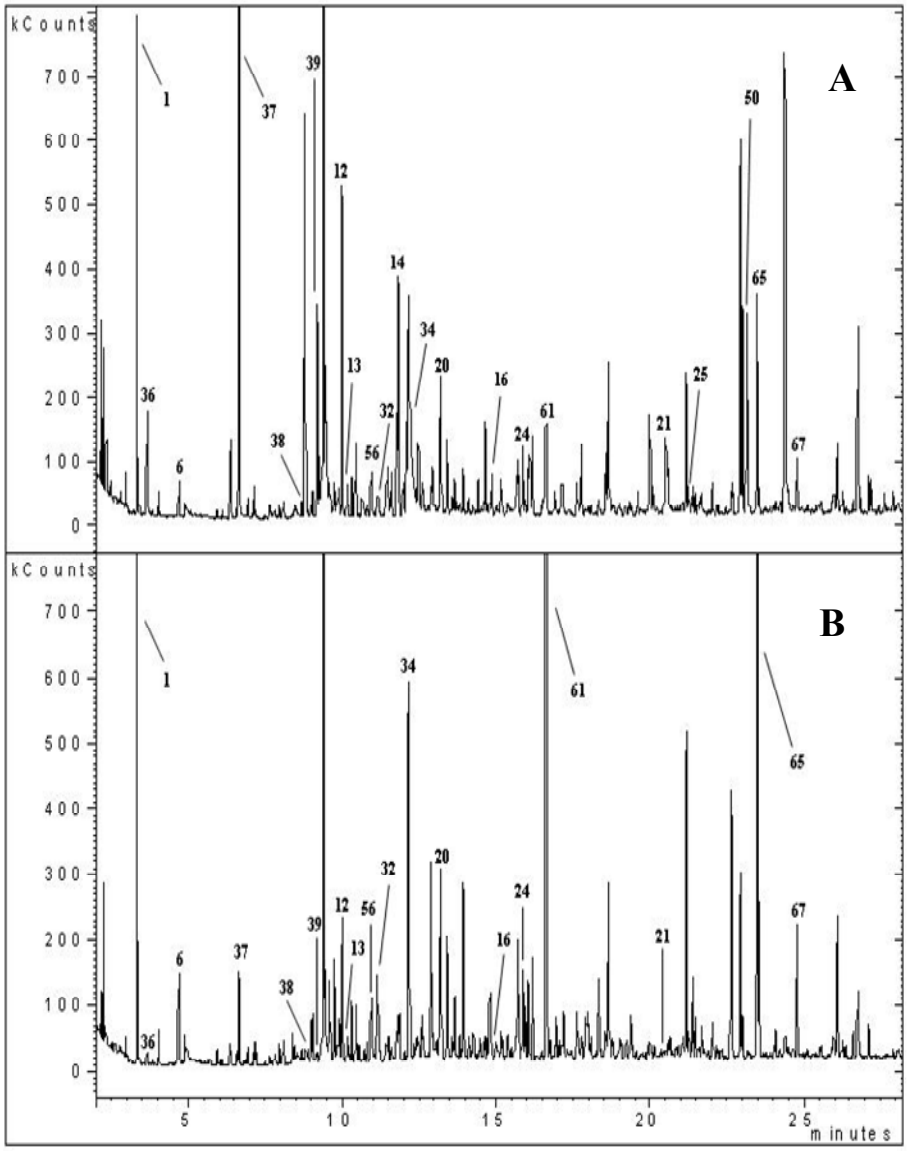


Figure 1. Chromatographic profile of the HS-SPME using Divinylbenzene/PDMS fibre analysis in S0int leaves (A) and S0ext leaves (B) of *Brassica oleracea* L. var. *costata* DC. Identity of compounds as in Table 2.

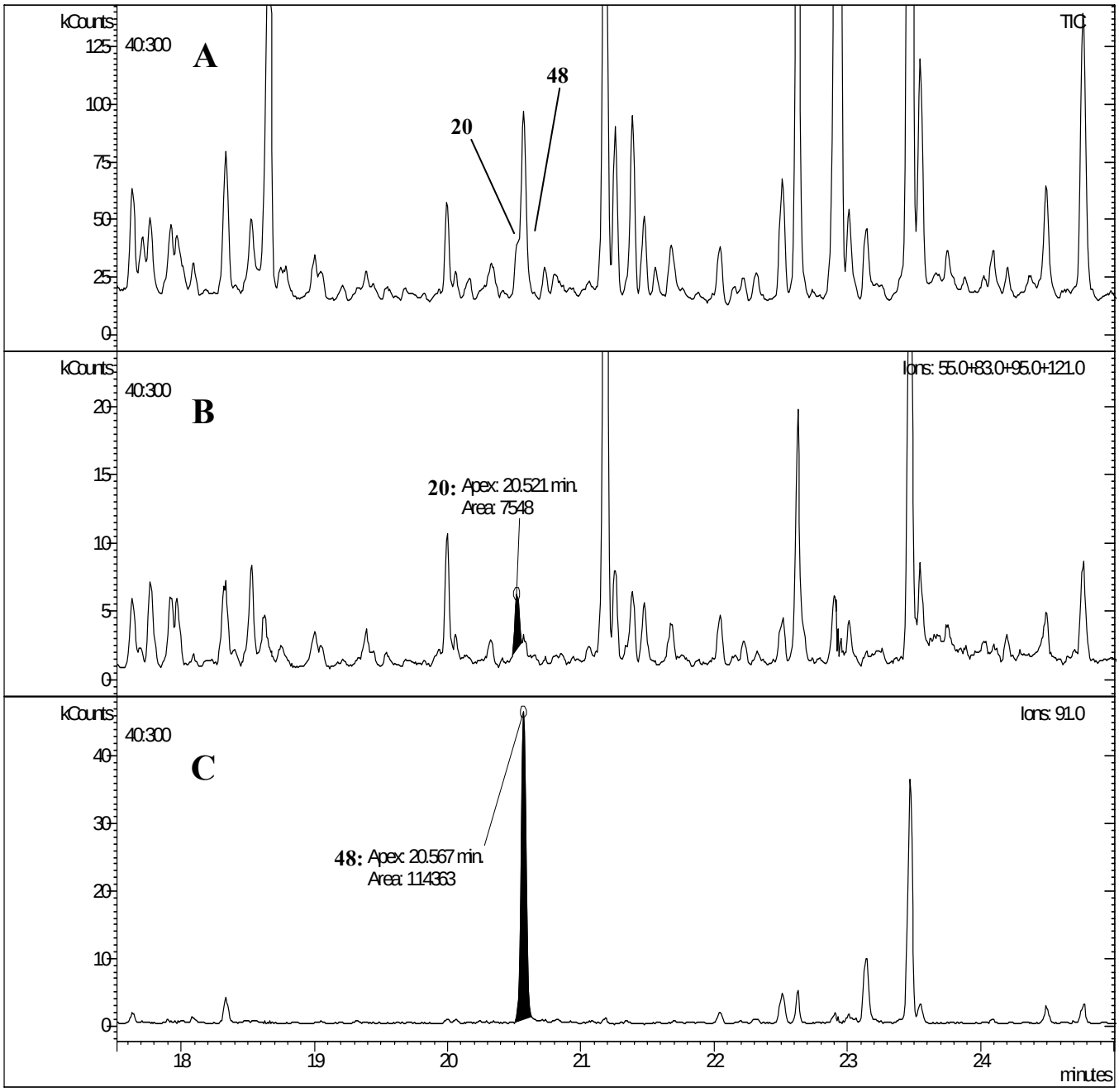
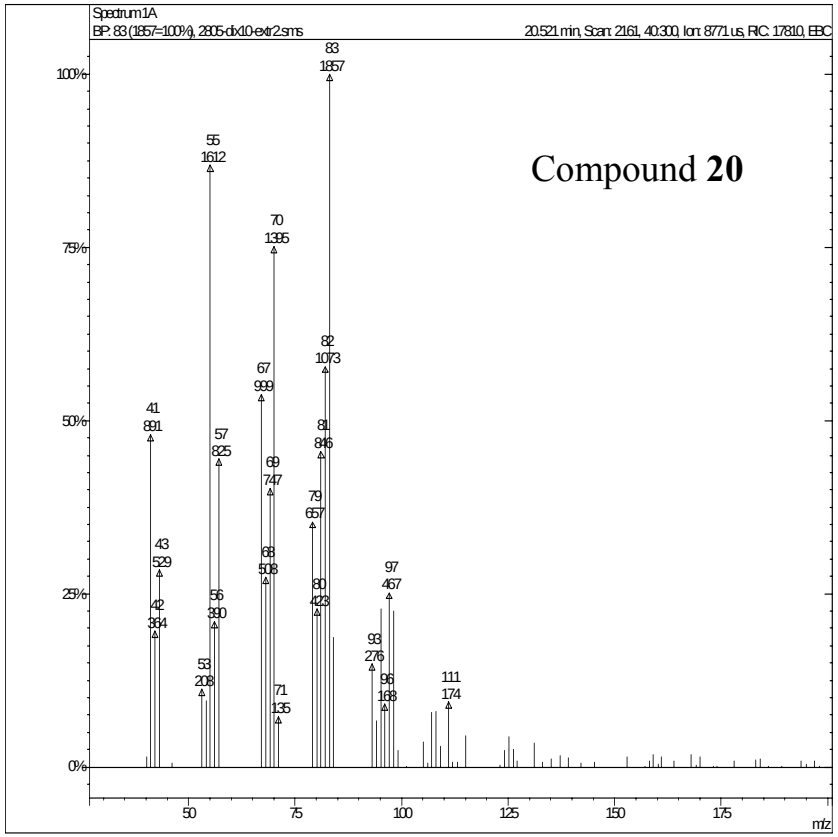


Figure 2a. Chromatograms of the HS-SPME of Dix10-ext leaves of *Brassica oleracea* L. var. *costata* DC, (A) Chromatogram in Full scan acquisition, (B) re-construcetd chromatogram by selected ion monitoring (*m/z*=55, 83, 95 and 121), scan=2161, ion=8771; RIC=38598 and retention time=20.521 min, and (C) re-constructed chromatogram by selected ion monitoring (*m/z*=91), Scan=2167, ion=3955; RIC=97100 and retention time=20.567 min.. Identity of compounds as in Table 2.



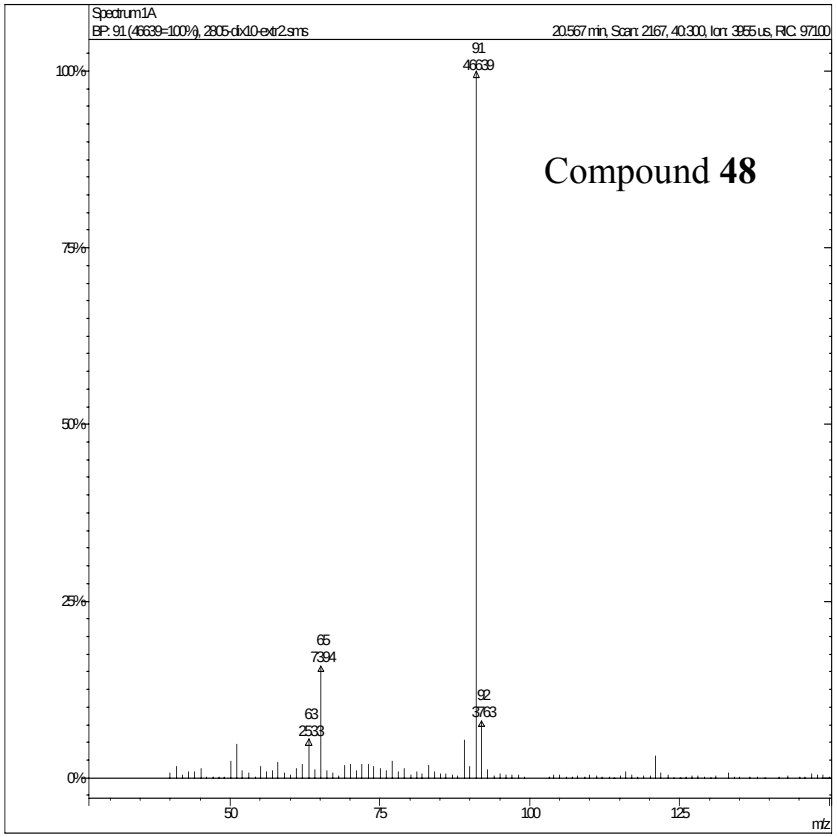


Figure 2b. Mass spectra obtained from compound 20 and compound 48, respectively.

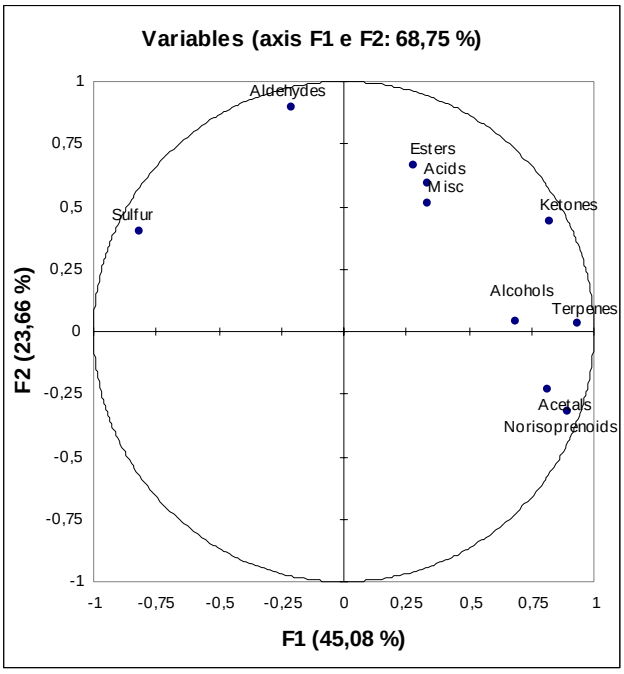


Figure 3. Projection of volatile compounds (variables: Norisoprenoids, Ketones, Aldehydes, Sulfur, Acetals, Alcohols, Esters, Terpenes, Acids, Misc-miscellaneous compounds) into the plan composed by the 2 principle axes F1 and F2. The 2 plans contain 68.75% of the total variance.

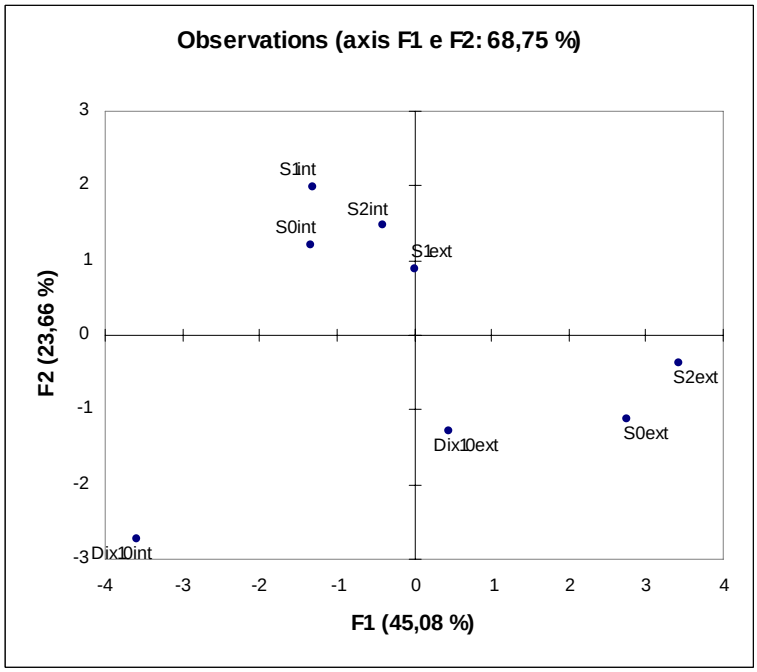


Figure 4. Projection of samples (S0ext, S0int, S1ext, S1int, S2ext, S2int, Dix10ext, Dix10int) into the two principal components.

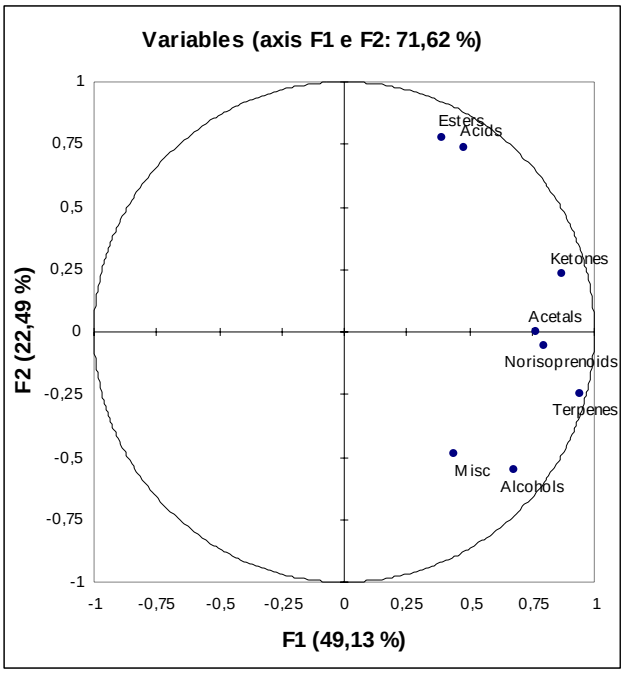


Figure 5. Projection of volatile compounds (variables: Norisoprenoids, Ketones, Acetals, Alcohols, Esters, Terpenes, Acids, Misc-miscellaneous compounds) into the plan composed by the 2 principle axes F1 and F2. The 2 plans contain 71.62% of the total variance.

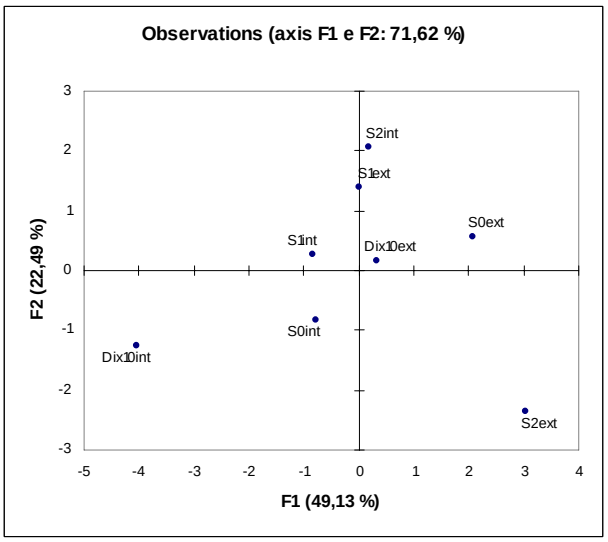


Figure 6. Projection of samples (S0ext, S0int, S1ext, S1int, S2ext, S2int, Dix10ext, Dix10int) into the two principal componentses of *Brassica olearacea* L. var. *costata* DC.

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4.11. Water extracts of *Brassica oleracea* var. *costata* potentiate paraquat toxicity to rat hepatocytes *in vitro*

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TITLE:

Water extracts of *Brassica oleracea* var. *costata* potentiate paraquat toxicity to rat hepatocytes *in vitro*

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ABSTRACT

Tronchuda cabbage extracts have been proven to have antioxidant potential against various oxidative species in cell free systems, though its antioxidant potential in cellular models remained to be demonstrated. In the present study, we used primary cultures of rat hepatocytes for the cellular assay system and paraquat PQ exposure as a pro-oxidant model agent, to test whether tronchuda cabbage water hydrolyzed extracts provide protective or aggravating effects towards PQ-induced oxidative stress and cell death. For this purpose cellular parameters related to oxidative stress were measured, namely the generation of superoxide anion, glutathione oxidation, lipid peroxidation, intracellular ATP levels, activation of nuclear factor-κB (NF-κB), activity of antioxidant enzymes, and cell death.

The obtained results demonstrated that the studied water hydrolyzed extracts of tronchuda cabbage, especially rich in kaempferol (84%) and other polyphenols, namely hydroxycinnamic acids and traces of quercetin, can potentiate the toxicity of PQ in primary cultures of rat hepatocytes. These results highlight that prospective antioxidant effects of plant extracts, observed *in vitro*, using non-cellular systems, are not always confirmed in cellular models, in which the concentrations required to scavenge pro-oxidant species may be highly detrimental to the cells.

Keywords: *Brassica oleracea* var. *costata*; primary cultured rat hepatocytes; paraquat; oxidative stress; toxicity.

1. Introduction

Epidemiological evidence demonstrates that diets rich in fruits and vegetables promote health, and attenuate or delay the onset of various diseases, including cardiovascular diseases, diabetes, certain cancers, and several age-related degenerative disorders (Cesar, 2007). These protective effects can be mostly attributed to antioxidant and/or anti-inflammatory activities exerted by dietary polyphenols, either directly or as a result of its interaction with various signalling pathways (Rahman *et al.* 2006). Actually, several polyphenols, particularly flavonoids, have the ability to react with pro-oxidant reactive species, without generating further intrinsic reactivity, therefore quenching chain reactions (Masella *et al.* 2005). In addition, flavonoids have been considered to possess *in vitro* and *in vivo* anti-inflammatory properties, resulting from their interactions with several key inflammatory enzymes, as well as signalling cascades involving cytokines and regulatory transcription factors (Gomes *et al.* 2008). However, while there is ample evidence that a flavonoid-rich diet may promote health and provide protection from age-related diseases, uncertainty remains regarding the conditions and the levels of flavonoid intake capable to pose a potential health hazard (Skibola and Smith, 2000). As it was observed with some plant extracts, they can be either protective or toxic, depending on extract concentrations and cellular conditions, under quiescent or stressful conditions (Lima *et al.* 2004).

Tronchuda cabbage water extract, a rich source of polyphenolic compounds, namely acylated and non acylated kaempferol heterosides, has been thoroughly studied for its *in vitro* antioxidant activity in various cell free systems, and has been shown to effectively scavenge superoxide anion and hydroxyl radicals, nitric oxide, peroxinitrite and, to a lesser extent, hypoclorous acid (Ferreres *et al.* 2006; Sousa *et al.* 2008b; Vrchovská *et al.* 2006). From these results, it could be expected that the intake of tronchuda cabbage water extracts provides some degree of protection against pro-oxidant insults. However, these assays do not reflect the cellular physiological conditions, due to the inexistent interactions of the extract components with endogenous macromolecules, lack of absorption barriers and biotransformation. The cellular assays are frequently

used to study the mechanism of action since they closely reflect antioxidant activity within a biological environment (Liu and Finley, 2005).

In the present study, we used primary cultures of rat hepatocytes for the cellular assay system and paraquat (PQ) exposure as a pro-oxidant model agent, to test whether tronchuda cabbage water extract provides protective or aggravating effects towards PQ-induced oxidative stress and cell death. For that purpose, several endpoints reflecting oxidative stress related damage and signalling were evaluated, namely the generation of superoxide anion, glutathione oxidation, lipid peroxidation, ATP levels, activation of nuclear factor-κB (NF-κB), activity of antioxidant enzymes, and cell death. The obtained results indicate that while a small protective effect may be achieved at low extract concentrations, PQ-induced toxicity is potentiated at flavonoid concentrations attained *in vivo*.

2. MATERIALS AND METHODS

2.1. Chemicals

Collagenase type I (EC 232-582-9), glutathione reductase (EC 1.6.4.2), and luciferase (EC 1.13.12.7) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Culture media and supplements were obtained from Lonza Ltd. (Basel, Switzerland). Collagen G was from Biochrom AG (Berlin, Germany). Synthetic oligonucleotides for NF-κB assay were from Amersham Pharmacia Biotech (Uppsala, Sweden). Other reagents were from Sigma and Merck (Darmstadt, Germany). All reagents were of analytical grade.

2.2. Plant material

The leaves of Tronchuda cabbage (*B. oleracea* var. *costata*) grown under organic fertilization were obtained from *CIMO/Escola Superior Agrária, Instituto Politécnico de Bragança*. Dark green leaves were selected, frozen at -20 °C and lyophilized. The dry material (3 g) was then boiled for 15 min in 600 mL of deionized water, cooled to room temperature, filtered through a Büchner funnel and freeze-dried.

Phenolic compounds in the extract are highly acylated and glycosylated and it is known that dietary flavonoids may become deglycosylated during passage across the small intestine (Day *et al.* 1998) or by bacterial activity in the colon, (Kim *et al*. 1998) , which facilitates their cellular uptake. Thus, the lyophilized crude extract (1.4 g) was subjected to a two step chemical hydrolysis. In brief, the extract was dissolved in 10 mL of 2 M NaOH and allowed to stand for 4 h at room temperature in the dark. The alkaline hydrolysis products were acidified with concentrated HCl to pH 1-2 and then eluted through an ISOLUTE C_{18} column (NEC, 50 μm particle size, 60 Å porosity; Sorbent Technology Ltd, Mid Glamorgan, UK), previously conditioned with methanol and acidified water (pH 1-2). Polar compounds were eluted with acidified water before the elution of phenolics with methanol. The methanolic extract was filtered, concentrated to dryness under reduced pressure at 40 °C and dissolved in 10 mL of 2 M HCl for acid hydrolysis. This solution was kept for 30 min in a water bath adjusted to 100 °C, cooled to room temperature and subjected to a purification process with a C_{18} column as described before. The dried hydrolysed extract obtained was dissolved in 5 mL of DMSO, sterile filtered and stored at -20 ºC.

2.3. Phenolic Content of Tronchuda Cabbage Extract

The polyphenol content of tronchuda cabbage extract before and after hydrolysis was measured by HPLC-DAD as previously described (Ferreres *et al.* 2005). The hydrolysed extract contained kaempferol (84%), sinapic acid (10%), ferulic acid (4%), caffeic and p-coumaric acid (1% each), and traces of quercetin, accounting for 1.2 mg/mL of polyphenolic compounds expressed as kaempferol equivalents (equivalent to 2.0 mg/g of dried extract). The non-hydrolysed extract was characterized by the presence of 13 kaempferol derivatives and 3-*p*-coumaroylquinic acid as previously reported, (Sousa *et al.* 2008a), accounting for 12.0 mg of kaempferol equivalents / g of dried extract.

2.4. Animals

Housing and experimental treatment of animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (Institute for Laboratory Animal Research, 1996).

Adult male Wistar rats (Charles-River Laboratories, Barcelona, Spain), weighing 250–300 g were used. For at least 1 week prior to use, animals were acclimatized in polyethylene cages, lined with wood shavings, with wire-mesh tops, at an ambient temperature of 20±2 °C, humidity between 40% and 60% and 12/12 h light/dark cycle (light on from 8.00 to 20.00 h), in our animal facilities, having standard chow and tap drinking water ad libitum. Surgical procedures for the isolation of hepatocytes were performed under anaesthesia and carried out between 10.00 A. M. and 11.00 A. M.

2.5. Isolation of rat hepatocytes

Isolation of hepatocytes was performed by a two-step collagenase perfusion as previously described (Pontes *et al.* 2008b). In brief, the liver from adult Wistar male rats was perfused *in situ via* portal vein with a calcium-free Hank's solution containing 0.67% albumin, 12.5 mM HEPES (pH 7.4) and 0.06 mM EGTA. The flow rate and temperature were set at 10 mL/min and 37 ºC, respectively. On the second step, the enzymatic digestion was performed with a similar buffer, without EGTA, and containing 0.05% collagenase and 0.44% (w/v) $CaCl_2$. The cells were dispersed in Krebs-Henseleit solution with 0.67% albumin and 12.5 mM HEPES, filtered through silk gauze and washed by low speed centrifugation (50 g) for 2 min and washed twice in Krebs – Henseleit solution with 12.5 mM HEPES to remove cell debris, damaged cells and nonparenchymal cells. The isolated hepatocytes were incubated on ice for 30 min with 500 U/mL penicillin G, 0.5 mg/mL streptomycin sulphate and 1.25 µg/mL amphotericin B. Upon isolation, the hepatocytes viability was >80% as estimated by trypan blue exclusion.

2.6. Cell culture and treatments

The isolated hepatocytes were plated on collagen-coated 6 or 12-well culture plates (7.5 and 3 $x10^5$ cells/well, respectively) in William's E medium (WME) supplemented with 5% FCS, 0.5 nM dexamethasone, 860 nM insulin, 2 mM l-glutamine, 10 mM HEPES, 100 U/ml penicillin G, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, and kept at 37°C in an atmosphere of 5% CO_2/ 95% air (Lima *et al.* 2006). Cells were allowed to attach for 3 h before switching to a serum-free medium, and then cultured for another 20 h before treatments.

To study the effect of tronchuda cabbage on PQ induced toxicity, hepatocytes were pre-incubated for 1 h with various concentrations of tronchuda cabbage hydrolysed extract (between 3 and 4000 μg/mL) or vehicle (DMSO 0.5 %). Cells were then incubated with PQ (10 mM) for 24 hours.

2.7. Measurement of Superoxide Radicals

Superoxide radicals were measured by the NBT reduction assay as described previously (Sharma and Mongan, 2001). In brief, NBT (5 mM final concentration) was added to the medium and incubated at 37 °C for 1 h. Then, the incubation medium was removed, the cells were lysed with DMSO: 4 mM KOH (1:1), and the absorbance of reduced NBT (formazan) was measured at 630 nm.

2.8. Lactate dehydrogenase (LDH) leakage assay

LDH activities were measured spectrophotometrically by a kinetic NADH oxidation assay at 340 nm (with background correction at 620 nm) as described previously (Santos-Marques *et al.* 2006). Briefly, after a 24 h treatment period, an aliquot of culture medium was taken to determine the activity of LDH leaked through cell membranes and the cell monolayer was lysed in the remaining medium by sonication to determine total LDH activity. Results are expressed as LDH activity in media, relative to total activity (medium plus cell lysate).

2.9. MTT reduction assay

Mitochondrial function was assessed by the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan as described by our group (Capela *et al.* 2007). After a 24 h treatment period, the medium was removed and the cells were incubated for 1 h at 37°C in WME containing 0.5 mg/mL MTT, followed by the addition of 10% SDS in 0.01 M HCl. The plates were incubated overnight to allow formazan solubilization and the absorbance was measured at 570 nm. Data are presented as the percentage of MTT reduction of treated cells relative to vehicle.

2.10. Measurement of ATP

ATP determination was based on the enzyme luciferase catalysis of the oxidative decarboxylation of luciferin in the presence of ATP and Mg^{2+} producing a bioluminescence signal (Pontes *et al.* 2008b). The light emitted is proportional to the ATP present when ATP is the rate limiting reagent. In this assay, ATP was extracted from cells with 0.5 M $HClO_4$ and, after neutralization with 0.76 M $KHCO_3$ the samples were mixed with luciferin-luciferase reagent (150000 U/mL luciferase and 40 μg/mL luciferin). The light emitted was read immediately using a microplate luminometer (BioTek Instruments, Vermont, US).

2.11. Measurement of total and oxidized glutathione

The cellular glutathione (GSH) levels were determined by the DTNB-GSSG reductase recycling assay after protein precipitation with perchloric acid as described before (Pontes *et al.* 2008b). Oxidized glutathione (GSSG) was determined after sample pretreatment with 2-vinylpyridine.

2.12. Measurement of lipid peroxidation

Lipid peroxidation was determination by the thiobarbituric acid-reactive substances assay, as previously described (Dinis-Oliveira *et al.* 2007) with some modifications. Hepatocytes ($1.5x10^6$) were washed with PBS, scrapped, pelleted by centrifugation, ressuspended in 500 μl PBS and sonicated. Then, 50 μL of 10% SDS and 325 μL of 0.5% (w/v) thiobarbituric acid in 20% (v/v) acetic acid (pH 3.5) were added. The reaction mixtures were placed in a water bath and heated for 60 min at 95–100 °C, cooled on ice, and then centrifuged at 1600 g for 10 min. The absorbance of the supernatants was read at 532 nm. Results were expressed as a percentage of thiobarbituric acid-reactive substances relative to control (vehicle).

2.13. Measurement of antioxidant enzymes activity

In order to measure enzyme activities, hepatocytes were washed with PBS, scrapped, pelleted by centrifugation, ressuspended in 0.1 M phosphate buffer

and sonicated. After centrifugation at 10000 g for 15 min the supernatant was collect and stored at -80 °C until enzyme assays. The enzyme activities were determined as previously described (Dinis-Oliveira *et al.* 2007).

Glutathione peroxidase (GPx) activity was measured indirectly by the coupled assay of NADPH oxidation using H_2O_2 as substrate. In this assay, oxidized glutathione produced upon reduction of 0.25 mM H_2O_2 by GPx is recycled to its reduced state by glutathione reductase (GR) accompanied by the oxidation of NADPH. The decrease in absorbance at 340 nm enables the quantification of GPx under conditions in which its activity is rate limiting.

Glutathione reductase (GR) activity was determined following the decrease of NADPH at 340 nm needed for the reduction of GSSG (0.3 mM) to GSH.

Catalase (CAT) activity was measured following the decomposition of 10 mM H_2O_2 at 240 nm.

2.14. Measurement of quinone reductase activity

Quinone reductase (QR) activity assay was based on the reduction of menadione to menadiol, coupled to the non-enzymatic reduction of MTT to formazan (Prochaska and Talalay, 1988; Uda *et al.* 1997). Briefly, 0.5% bovine serum albumin, 0.025% Tween-20 (v/v), 0.36 mM NADPH, 0.3 mg/mL MTT and 25 μM menadione in 25 mM Tris–HCl (pH 7.4) were added to the sample prepared as described above, (except that a Tris-HCl buffer was used) and the increase in absorbance at 610 nm was measured. In order to determine non specific enzyme activity, a reaction mixture containing 0.3 mM dicoumarol in 5 mM potassium phosphate buffer pH 7.4 was tested. The reaction rate obtained was subtracted to that obtained in the absence of dicoumarol.

2.15. Extraction of nuclear proteins

A modified procedure based on the method previously described by us (Dinis-Oliveira *et al.* 2007) was used to extract nuclear proteins. The cell layers ($1.5x10^6$) were washed twice with ice-cold PBS, scrapped and spun down. The cell pellets were lysed with 200 μL of hypotonic buffer (10 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 2 mg/mL aprotinin, 10 mg/mL leupeptin, 5

mg/mL pepsatin A , 5 mM NaF, 1 mM $NaVO_4$). After 10 min incubation on ice, igepal (0.5% final concentration) was gently mixed and nuclei were pelleted by centrifugation at 8000 x *g* for 15 s. The supernatant was removed, and the pellet was resuspended in 50 μl of hypertonic buffer (20 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM EGTA, 420 mM NaCl, 20% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 2 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/ml pepsatin A, 5 mM NaF, 1 mM $NaVO_4$) and incubated for 30 min on ice with gentle mixing. Nuclear proteins were obtained by centrifugation at 16000 g for 15 min and aliquots of the collected supernatants, containing the nuclear proteins, were stored at -80 °C.

2.16. NF-κB activation assay

The NF-κB activation was evaluated using an electrophoretic mobility shift assay (EMSA) (Dinis-Oliveira *et al.* 2007). Briefly, 10 μg of nuclear protein extract were incubated overnight at 4 °C with 4 pmol Cy5 labelled double stranded oligonucleotide (consensus sequence 5' - GCC TGG GAA AGT CCC CTC AAC T – 3'), 1 μg of poly(dI.dC), 7.5% glycerol, 0.3% Igepal, 1 mM EDTA, 75 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 16 mM HEPES, pH 7.9, in a final volume of 20 μL. The incubation mixture was applied to a 5% polyacrylamide gel. Gels were run at 50 mA in TBE buffer (45 mM Tris-borate, 10 mM EDTA). Analysis of competition with unlabeled oligonucleotides was performed using a 50-fold excess of double stranded DNA in the reaction mixtures. For supershift experiments, the reaction mixtures were incubated on ice for 30 min in the presence of 1 μL of specific polyclonal antibody against NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA).

2.17. Measurement of protein content

Protein content was measured as previously described using bovine serum albumin as a standard (Pontes *et al.* 2008a).

2.18. Statistical analysis

Statistical significance of differences between control and treatment groups was determined using one-way analysis of variance (ANOVA), with the Bonferroni

post hoc test. Results represent the mean ± standard error of the mean (SEM) of at least four experiments. Each experiment was performed in a cell culture prepared from a different hepatocyte isolation procedure. P values less than 0.05 were considered significant.

3. Results

3.1. PQ induces concentration-dependent toxicity in primary rat hepatocytes

Primary cultured rat hepatocytes were exposed to PQ concentrations ranging from 0 to 20 mM for 24 h, and assayed for cell viability (LDH leakage), mitochondrial function (MTT, ATP), and biomarkers of oxidative stress (GSH, GSSG and lipid peroxidation), in order to establish the PQ levels that result in toxic effects to the cells.

The results showed a concentration-dependent increase of LDH leakage, which was significant for the two highest concentrations tested (Fig 1a).

Figure 1b shows that the levels of MTT reduction were significantly decreased at the lowest PQ concentration tested and were further decreased in a concentration-dependent manner (Fig. 1b).

ATP depletion was significant for PQ concentrations of 10 mM (7.7 nmol ATP/ mg protein compared with 12.4 nmol / mg protein in control samples). When cells were treated with 20 mM PQ, ATP levels dramatically declined to 16% of control samples (Fig. 1c).

PQ had no significant effect on total glutathione, but the oxidized glutathione (GSSG) increased in a concentration-dependent manner, attaining significance at 10 mM (3.9 nmol / mg protein compared to 0.97 nmol / mg protein for the control) (Table 1). The ratio of GSSG/(GSH + GSSG) increased for all PQ concentrations tested, being significantly higher than the control at 10 and 20 mM of PQ (Fig. 1d). A concentration of 10 mM PQ significantly increased lipid peroxidation in primary rat hepatocytes (Table 2).

Based on these results, a concentration of 10 mM PQ was chosen for all the subsequent assays to evaluate the antioxidant or pro-oxidant effects of tronchuda cabbage extracts.

3.2. Tronchuda cabbage extracts are not cytotoxic

The plant extract by itself did not exert any toxic effects on hepatocytes over the concentration range tested (between 3.1 and 4000 μg/mL of hydrolysed extract) as ascertained by the results for LDH leakage (10.7 ± 0.7 % at 4000 μg/mL, *vs* 8.3 ± 0.7 % for control samples), MTT (higher than 92% for all extract concentrations), ATP (ranging from 12.4 ± 0.8 in control samples to 16.2 ± 2.0 nmol/mg protein at 4000 μg/mL), total glutathione (the values increased from 13.4 ± 0.9 nmol/mg protein for the control samples to 17.0 ± 3.0 nmol/mg protein at 800 μg/mL and decreased to 10.1 ± 2.9 nmol/mg protein at 4000 μg/ml of extract) and GSSG (with similar values for control and all extract concentrations) (Table 3).

3.3 Effect of cabbage extract on PQ induced toxicity in primary rat hepatocytes

To evaluate the effect of tronchuda cabbage hydrolysed water extracts on PQ induced toxicity, primary rat hepatocytes were pre-treated for 1 hour with extract concentrations ranging from 3.1 to 4000 μg/mL and then exposed for 24 hours to 10 mM PQ. The effects on LDH, MTT, ATP, total glutathione and GSSG were evaluated. The pre-treatment with extract concentrations between 3.1 and 800 μg/mL did not significantly change the effect of 10 mM PQ in these parameters (Fig. 2, a-d). For higher extract concentrations, differences between hepatocytes treated only with PQ and hepatocytes treated both with extract and PQ were observed. LDH leakage was significantly increased compared to only PQ treated samples, for extract concentrations above 1200 μg/mL, reaching a plateau around 85% at the concentration of 1600 μg/mL (Fig. 2a). The same profile was observed with MTT reduction, which reached a plateau of around 7% reduction at 1200 μg/mL (Fig. 2b). ATP depletion was aggravated by the extract at concentrations of 800 μg/mL and further decreased at higher extract concentrations, being significantly different from PQ exposed samples at 1600 μg/mL (Fig. 2c). The ratio between oxidized and total glutathione is depicted in figure 2d. Due to the high mortality of hepatocytes, the oxidized glutathione levels were not measured for hepatocytes treated with extract concentrations above 800 μg/mL. Total glutathione depletion became evident at 2400 μg/mL

(7.0 ± 1.8 nmol/mg protein compared to 13.4 ± 0.9 nmol/mg for control samples and 14.2 ± 1.4 nmol/mg for PQ treated samples).

Overall, these results showed that 200 μg/mL was the highest extract concentration that did not increase PQ toxicity for any of the parameters measured. For this reason, the effect of the pre-treatment of hepatocytes with 200 μg/mL of tronchuda cabbage extract was used to measure ROS levels, lipid peroxidation, antioxidant enzymes activity and NF-κB activation in hepatocytes exposed to 10 mM PQ.

The generation of ROS by hepatocytes exposed to PQ and the possible scavenging of tronchuda cabbage extract was verified by the addition of NBT to the culture medium. After 24 h incubation, NBT reduction was doubled on PQ exposed hepatocytes (Table 2). The results of NBT reduction did not change significantly when hepatocytes were pre-treated with 200 μg/mL of tronchuda cabbage extract. The superoxide levels in cells treated only with 200 μg/mL tronchuda cabbage were similar to control samples.

Lipid peroxidation measured as thiobarbituric reactive substances (TBARS), was increased by 83% with PQ exposure (Table 2) and by 46% when the hepatocytes were pre-treated with the extract, though no significant differences were found between the two groups.

To assess the potential role of antioxidant enzymes in PQ induced toxicity, catalase, GPx and GR were analysed. QR activities of PQ exposed hepatocytes was also measured (Table 2). Under the assay conditions, catalase was the only enzyme that significantly increased after PQ exposure (23%) and this effect was reversed by extract pre-treatment. QR was induced by tronchuda cabbage extract (53 %), but this induction was not observed when hepatocytes were treated with tronchuda cabbage and PQ.

As it can be seen in figure 3, 10 mM PQ strongly activated NF-κB in hepatocytes after 1 h exposure and this effect was reversed by the extract pre-treatment, being this effect more profound on band 3. The extract itself slightly decreased the NF-κB band compared to control hepatocytes. Specificity of bands was confirmed by the competition assay and supershift assays using p50 and p65 antibodies (data not shown).

3.5 Effect of tronchuda cabbage extract on Quinone Reductase activity

In a parallel set of experiments, hepatocytes were tested for the induction of QR with tronchuda cabbage extracts. As shown in figure 4, the tronchuda cabbage extract-mediated induction of QR activity was strong and concentration dependent, reaching 73.6 ± 9.9 % when tested with 800 µg/mL of extract (5.6 µM kaempferol equivalents). When tested at higher concentrations tronchuda cabbage extracts did not further induce QR (data not shown).

Table 1 - Effect of PQ on total and oxidized glutathione content, in primary rat hepatocytes, after 24 h treatment.

PQ (mM)	0	2.5	5	10	20
GSH + GSSG (nmol/mg prot)	13.4 ± 0.93	15.6 ± 0.69	14.8 ± 0.54	14.2 ± 1.36	10.1 ± 0.94
GSSG (nmol/mg prot)	0.97 ± 0.044	1.9 ± 0.56	2.9 ± 0.65*	3.9 ± 0.46***	4.0 ± 0.46***

Values are given as mean ± SEM (n = 5). $^{*}P < 0.05$, and $^{***}P < 0.001$ versus control group.

Table 2 - NBT oxidation, TBARS, antioxidant enzymes and Quinone reductase activity measured on hepatocytes exposed to PQ (10 mM); extract (200 µg/mL) and extract + PQ.

	PQ	Extract	Extract + PQ
NBT	203.7 ± 18.20^{aaa}	100.6 ± 6.76^{bbb}	195.2 ± 6.77$^{aaa, ccc}$
TBARS	183.1 ± 18.68 ^{aa}	123.4 ± 11.77	145.6 ± 19.14
Catalase	123.0 ± 3.75^{a}	87.06 ± 4.52^{bbb}	94.7 ± 8.89^{bb}
GPx	108.8 ± 7.23	105.4 ± 4.78	106.5 ± 8.23
GR	96.01 ± 3.41	106.8 ± 5.41	106.6 ± 6.46
QR	85.6 ± 7.65	153.3 ± 5.13$^{aaa, bbb}$	111.6 ± 6.11$^{b, ccc}$

Values (percentage) are given as mean ± SEM (n = 4). $^{a}P < 0.05$, $^{aa}P < 0.01$, and $^{aaa}P < 0.001$ versus control group; $^{b}P < 0.05$, $^{bb}P < 0.01$ and $^{bbb}P < 0.001$ versus PQ group; $^{c}P < 0.05$, $^{cc}P < 0.01$ and $^{ccc}P < 0.001$ versus extract group.

Table 3 - Tronchuda cabbage extracts effects on LDH leakage, MTT reduction, ATP and glutathione (total and oxidized) after 24 h hepatocytes treatment.

Extract (mg/mL)	LDH leakage (%)	MTT reduction (% of control)	ATP (nmol/mg prot)	GSH + GSSG (nmol/mg prot)	GSSG (nmol/mg prot)
0	6.5 ±1.53		12.4 ± 0.93	13.4 ± 0.51	1.0 ± 0.05
3.1	5.8 ± 0.84	96.3 ± 2.13	12.7 ± 0.95	13.3 ± 1.92	0.9 ± 0.04
12.5	6.4 ± 0.95	97.6 ± 1.49	12.6 ± 1.02	14.4 ± 1.65	0.9 ± 0.05
50	6.2 ± 1.13	98.7 ± 0.96	12.4 ± 0.97	14.9 ± 2.03	1.0 ± 0.06
200	6.3 ± 2.30	91.9 ± 1.55	13.2 ± 1.12	15.7 ± 2.62	1.0 ± 0.03
800	4.2 ± 0.70	97.4 ± 1.15	12.9 ± 1.14	17.0 ± 3.02	1.0 ± 0.09
1600	6.1 ± 1.14	101.9 ± 5.72	13.9 ± 1.00	17.0 ± 3.21	0.9 ± 0.07
2400	8.8 ±0.67	98.6 ± 2.95	14.3 ± 1.26	15.7 ± 3.38	0.9 ± 0.09
3200	8.6 ± 0.96	103.5 ± 5.78	15.3 ± 0.99	13.6 ± 2.63	1.0 ± 0.17
4000	10.7 ± 0.68	98.07 ± 9.57	16.2 ± 1.98	10.1 ± 2.90	1.0 ± 0.12

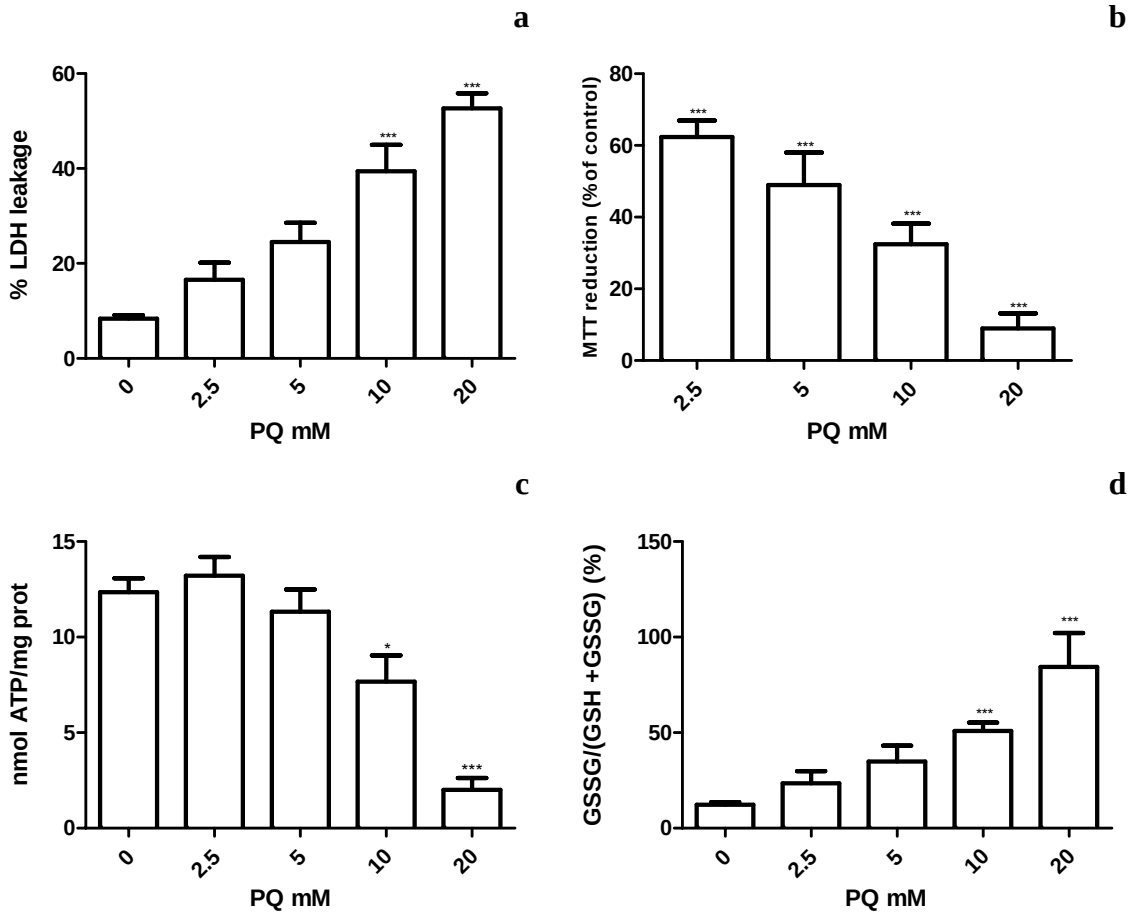

Fig. 1. Concentration-effect curves for PQ on primary rat hepatocytes after 24 h exposure (n = 5). **a.** % LDH leakage, **b**. % of MTT reduction relatively to control hepatocytes; **c**. ATP; **d**. % GSSG/GSH; GSSG in GSH units. * compared to control hepatocytes. $^{*}P < 0.05$ and $^{***}P < 0.001$.

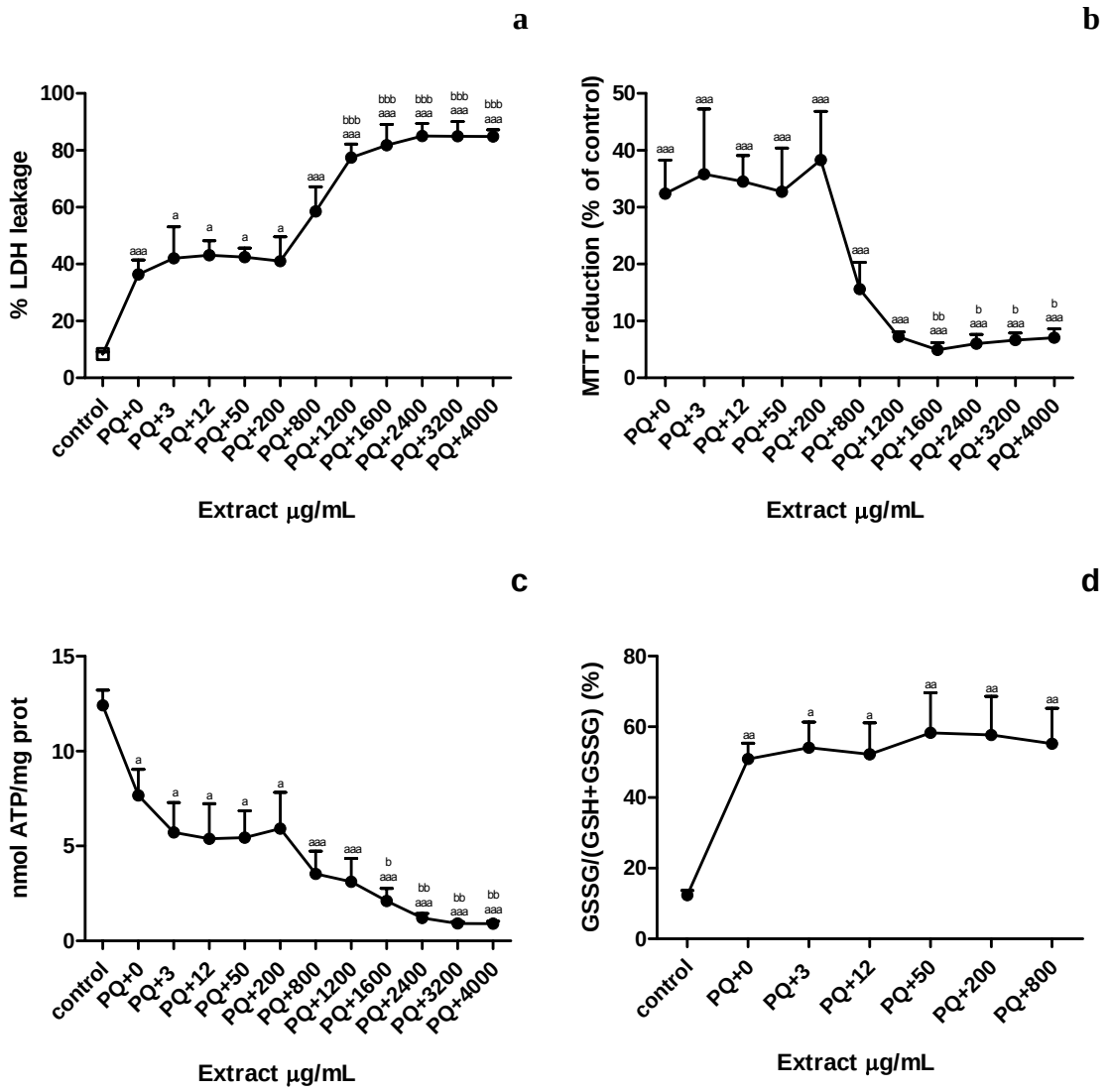

Fig. 2. Effect of tronchuda cabbage extract pre-treatment on PQ induced toxicity (n = 4). **a.** % LDH leakage, **b**. % of MTT reduction relatively to control hepatocytes; **c**. ATP; **d**. % GSSG/GSH. ^{a} compared to control hepatocytes; ^{b} compared to PQ exposed hepatocytes. ^{a} $P < 0.05$, ^{aa} $P < 0.01$, and ^{aaa} $P < 0.001$; ^{b} $P < 0.05$, ^{bb} $P < 0.01$ and ^{bbb} $P < 0.001$.

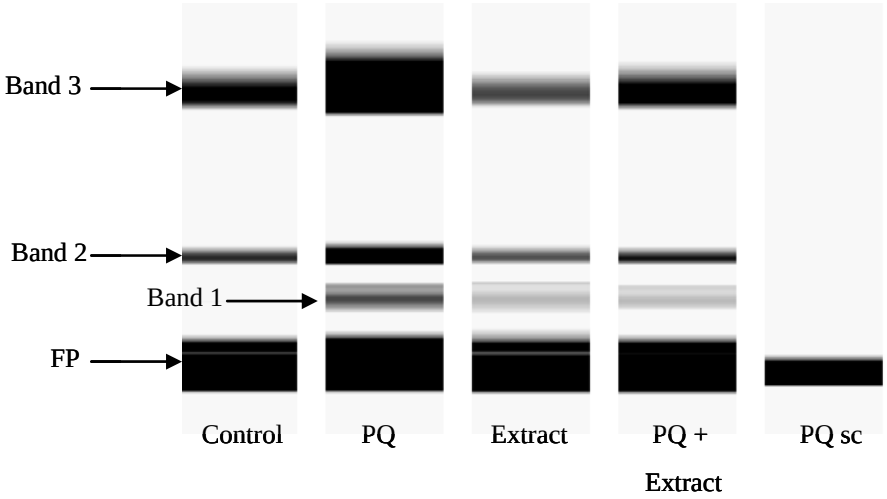

Fig. 3. fEMSA gel view of NFkB binding activation induced by 1h PQ exposure (n = 4). sc. specific competitor; FP. Free probe

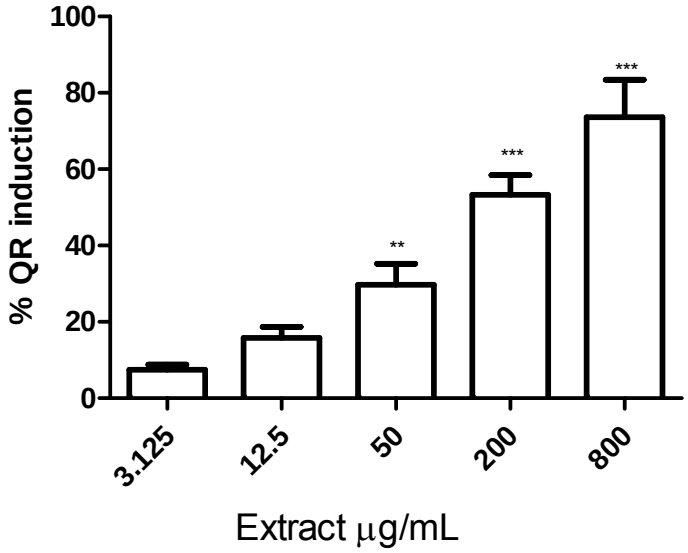

Fig. 4. Quinone reductase induction by tronchuda cabbage extracts after 24 h exposure (n = 4). * compared to control hepatocytes. $^{**}P < 0.01$ and $^{***}P < 0.001$

4. Discussion

The results obtained in the present study highlight that polyphenol – rich edible plant extracts are not always beneficial towards pro-oxidant damaging effects. Indeed, the antioxidant properties of tronchuda cabbage, previously observed in non-cellular assays (Ferreres *et al.* 2006; Sousa *et al.* 2008b; Vrchovská *et al.* 2006), and the results obtained in the present biological assay, showed lack of correlation. Although some degree of protection was obtained for the 200 µg/mL extract, an obvious potentiation of PQ-induced toxicity in the primary culture of rat hepatocytes was observed at higher concentrations.

The observed potentiation of PQ-induced toxicity was not related to possible toxic effects of the plant extract at the higher concentrations tested. Although no liver disorders related to tronchuda cabbage consumption have been reported, the concentrations used in this experimental assay with hepatocytes, were tested for possible detrimental effects. Over the concentration range tested (between 3.1 and 4000 µg/mL extract, corresponding to 77.5 to 100000 µg/mL cabbage fresh weight), no significant differences from control hepatocytes were detected on LDH, MTT, ATP, GSH and GSSG. The positive trend for an increase on ATP production with the extract concentration can be attributed to kaempferol-mediated stimulation of mitochondrial calcium uniporter, which leads to an increase in the respiratory rate and ATP production (Montero *et al.* 2004). Kaempferol, the major polyphenolic compound present in the extract, is known to induce toxicity in hepatocytes at high levels, with an LC_{50} of 1 mM after 2 h incubation (Moridani *et al.* 2002). Yet, as the most concentrated tronchuda cabbage extract tested contained only 28 µM kaempferol equivalents, the obtained results fit well with the expected low toxicity of the extract. These concentrations are more likely to be attained *in vivo* since flavonoids are known to be poorly absorbed. It was previously shown that the dietary intake of quercetin and kaempferol, two major flavonols in vegetables and fruits, can result in a plasma concentration of 100 nM (considering free and conjugated forms) (de Vries *et al.* 1998; DuPont *et al.* 2004; Sampson *et al.* 2002).

PQ was chosen to generate pro-oxidant deleterious conditions in rat hepatocyte cultures, as it is a widespread used and accepted model for inducing cellular

injury *via* oxidative stress. The redox cycling of PQ results predominantly in the production of superoxide anion. More toxic reactive oxygen species (ROS) and reactive nitrogen species (RNS) are subsequently generated, leading to oxidative stress and ultimately to cell death and inflammation-related fibrosis (Dinis-Oliveira *et al.* 2008). Thus, as expected, GSH oxidation, lipid peroxidation and cell death were observed in the present study, using cultured rat hepatocytes.

The ultimate effect of ROS and RNS on cells is dependent on a number of factors, including the level of oxidative stress generated, the nature of the intracellular signaling pathways activated, and the state of cellular antioxidant defences (Dinis-Oliveira *et al.* 2008). In an *in vivo* assay previously published by our group (Dinis-Oliveira *et al.* 2007) a strong activation of NF-κB was demonstrated in the lung of PQ exposed rats. In the present assay, a strong NF-κB activation in primary rat hepatocytes was also observed 1 hour after PQ exposure. Hepatocytes pre-treatement with only 200 μg/mL extract did not alter NF-κB binding activity but inhibited PQ-induced activation of NF-κB. This is in accordance with previously reported results showing that phenolic compounds commonly found in plants inhibit NF-κB activation in HepG2 cells treated with H_2O_2 (Musonda and Chipman, 1998). This effect may be related to the anti-inflammatory properties of flavonoids (Moroney *et al.* 1988). However, it is important to note that prolonged inhibition of NF-κB activity may be detrimental, since it is involved in the regulation of immune and defence genes necessary to overcome cellular insults (Musonda and Chipman 1998). NF-κB has been implicated in the inducible expression of genes related to oxidative stress, including GPx and CAT (Cheng *et al.* 1999; Rohrdanz and Kahl, 1998) and SOD (Lenardo and Baltimore, 1989) necessary to detoxify the ROS generated by pro-oxidant conditions, such as those induced by PQ. In accordance, Röhrdanz and collaborators showed that sub-cytotoxic doses of PQ (0.5 mM) induced catalase and SOD mRNA expression in primary rat hepatocytes (Rohrdanz *et al.* 2000). In the present study, catalase activity suffered a slight increase with PQ exposure, which was inhibited in the presence of the plant extract.

When hepatocytes were treated with 200 µg/mL extract and PQ no significant differences from control cells were seen on lipid peroxidation (table 2). Tronchuda cabbage extract antioxidant properties in vitro were previously demonstrated and correlated with its high polyphenolic content (Ferreres et al. 2006; Sousa et al. 2008b; Vrchovská et al. 2006). As antioxidants, flavonoids have shown to inhibit lipid peroxidation induced by various pro-oxidants in liver homogenates, microsomes, mitochondria and liposomes (Rodriguez et al. 2001). Kaempferol, the major flavonol detected in tronchuda cabbage has probably an important role in the observed scavenging effects. Upon reaction with ROS, kaempferol forms a phenoxyl radical that can originate quinone methide reactive intermediates (Moridani et al. 2002). The detoxification of the latter by QR implies NADH or NADPH consumption. Considering that the exposure of hepatocytes to PQ leads to depletion of NADH and NADPH, necessary for the reduction of PQ to the PQ free radical monocation (PQ.+) by cellular reductases (DeGray et al. 1991; Tampo et al. 1999), the detoxification of quinoic compounds became compromised. It can be assumed that the action of kaempferol as an antioxidant did not contribute to protect hepatocytes from PQ induced toxicity due to the lack of NADPH equivalents necessary to detoxify the product of kaempferol oxidation by PQ-generated ROS. The ability of kaempferol to induce QR, as we confirmed, reinforces the role of this enzyme in the detoxification of kaempferol or its derived metabolites. In a comparative study performed in an hepatocyte cell line (Hepa1c1c7 cells) with eight flavonoids, kaempferol was one of the most effective inducers of quinone reductase activity, with an approximately 2.5 fold increase when tested in a concentration of 20 µM (Uda et al. 1997).

In addition, flavonoids like quercetin or kaempferol can act as antioxidants or pro-oxidants depending on the assay conditions (Musonda and Chipman, 1998), and kaempferol, which contains a phenol ring, can be more pro-oxidant than quercetin, containing a catechol ring (Galati *et al.* 2002). Thus, flavonoids also have the potential to produce ROS and to collapse mitochondrial membrane potential, which has been considered the major hepatotoxic mechanism of these compounds (Galati *et al.* 2006; Galati *et al.* 2000; Moridani *et al.* 2002; Musonda and Chipman, 1998). Nevertheless, pro-oxidant effects can also be beneficial, since, by imposing a mild degree of oxidative stress the

levels of antioxidant defences and xenobiotic metabolising enzymes might be raised, leading to overall cytoprotection (Fahey and Kensler, 2007). These effects on cellular function can result from the interaction of flavonoids with various signaling pathways (Williams *et al.* 2004). It is noteworthy that flavonoids induce electrophile-responsive element (EpRE) via their pro-oxidant activity, which results in the expression of enzymes, such as QR, as noted above, and glutathione-S-transferases, which are major defense enzymes against electrophilic toxicants and oxidative stress (Lee-Hilz et al. 2006). In the present work, primary rat hepatocytes treated with tronchuda cabbage alone also showed induction of QR activity, as expected, considering its composition in polyphenolic compounds. Furthermore, *in vivo*, the presence of much higher concentrations of other low molecular weight antioxidants such as ascorbic acid and α-tocopherol and the blockade of radical-scavenging phenolic hydroxyl groups upon metabolization of flavonoids to methylated and glucuronidated forms, makes improbable that flavonoids act as major direct antioxidants in cells (Williams *et al.* 2004).

In conclusion, this work has clearly shown that the studied water extracts of tronchuda cabbage, especially rich in kaempferol (84%) and other polyphenols, namely, sinapic acid (10%), ferulic acid (4%), caffeic and p-coumaric acid (1% each), and traces of quercetin, can potentiate the toxicity of PQ in primary cultures of rat hepatocytes at concentrations higher than 200 µg/mL. These results are a strong reminder that prospective antioxidant effects of plant extracts, observed *in vitro*, using non-cellular systems, are not always confirmed in cellular systems, in which the concentrations required to scavenge pro-oxidant species may be highly detrimental to the cells.

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List of abbreviations

ATP – Adenosine-5'-triphosphate

CAT - Catalase

GPx – Glutathione Peroxidase

GR – Glutathione reductase

GSH – Reduced glutathione

GSSG – Oxidised glutathione

LDH – Lactate dehydrogenase

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NADH - β-nicotinamide adenine dinucleotide

NADPH - β-nicotinamide adenine phosphate dinucleotide

NF-κB – Nuclear factor kappa B

NBT - Nitro blue tetrazolium

PMS – Phenazine methosulfate

PQ – Paraquat

QR – NAD(P)H Quinone reductase

ROS – Reactive oxygen species

SOD – Superoxide dismutase

TBARS – Thiobarbituric acid – reactive substances

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4.12. Inflorescences of Brassicacea species as source of bioactive compounds: A comparative study

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Inflorescences of Brassicacea species as source of bioactive compounds: A comparative study

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Antimicrobial activity

ABSTRACT

Two *Brassica oleracea* varieties (*B. oleracea* L. var. *costata* DC and *B. oleracea* L. var. *acephala*) and *Brassica rapa* L. var. *rapa* inflorescences were studied for their chemical composition and antioxidant capacity. Phenolic compounds and organic acids profiles were determined by HPLC–DAD and HPLC–UV, respectively. *B. oleracea* var. costata and *B. oleracea* L. var. *acephala* inflorescences presented a similar qualitative phenolic composition, exhibiting several complex kaempferol derivatives and 3-*p*-coumaroylquinic acid, while *B. rapa* var. *rapa* was characterized by kaempferol and isorhamnetin glycosides and several phenolic acids derivatives. *B. oleracea* L. var. *costata* and *B. rapa* var. *rapa* showed the highest phenolics content. The three *Brassica* exhibited the same six organic acids (aconitic, citric, pyruvic, malic, shikimic and fumaric acids), but *B. oleracea* L. var. *acephala* presented a considerably higher amount. Each inflorescence was investigated for its capacity to act as a scavenger of DPPH radical and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid), exhibiting antioxidant capacity in a concentration dependent manner against all radicals. These samples were also studied for its antimicrobial potential against Gram-positive and Gram-negative bacteria and fungi, displaying antimicrobial capacity only against Gram-positive bacteria.

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1. Introduction

Brassica vegetables belong to the Cruciferous family, which includes a variety of economically significant horticultural crops. They are consumed all over the year as ingredients of different salads or after cooking of raw and frozen vegetables (Podsędek, 2007). Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC), kale (*B. oleracea* L. var. *acephala* DC) and turnip (*Brassica rapa* var. *rapa* L.) appear within the most consumed species.

Increasing attention has been paid to the role of diet in human health. In fact, food provides not only essential nutrients needed for life, but also other bioactive compounds for health promotion and disease prevention. It is generally assumed that the beneficial effects of vegetables are partly attributed to the complex mixture of phytochemicals possessing antioxidant activity (Liu, 2003; Podsędek, 2007). These comprise both phenolic compounds and organic acids (Liu, 2003; Podsędek, 2007; Pulido, Bravo, & Saura-Calixto, 2000; Silva et al., 2004), which contribute to their organoleptic features (Vaughan & Geissler, 1997), despite being applied in the quality control of several matrices (Fernandes et al., 2007; Ferreres et al., 2005; Sousa et al., 2005). Furthermore, plant compounds are known for their antimicrobial capacity (Cowan, 1999; Tim Cushnie & Lamb, 2005), which may be relevant considering the existing problem of resistance to antimicrobial agents.

The polyphenol composition of several materials from members of *Brassica* genus, or their byproducts, has been described (Llorach, Gil-Izquierdo, Ferreres, & Tomás-Barberán, 2003; Romani, Vignolini, Isolani, Ieri, & Heimler, 2006; Vallejo, Tomás-Barberán, & Ferreres, 2004), including that of *B. oleracea* var. *costata* (Ferreres et al., 2007, 2006, 2005; Sousa et al., 2005), *B. oleracea* var. *acephala* (Heimler, Vignolini, Dini, Vincieri, & Romani, 2006; Romani et al., 2003) and *B. rapa* var. *rapa* (Fernandes et al., 2007; Liang et al., 2006), referring distinct profiles between them. Recent publications also report the organic acids (Ayaz et al., 2006; Fernandes et al., 2007; Ferreres et al., 2007, 2006; Sousa et al., 2005) and the antioxidant potential (Fernandes et al., 2007; Ferreres et al., 2007, 2006; Heimler et al., 2006; Vrchovská et al., 2006) of these three species. However, information regarding their inflorescences is almost non-existent. As far as we know, only one study about the phenolic compounds and organic acids composition of *B. rapa* var.

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rapa was performed by our group, suggesting that this constitutes an interesting dietary source of protective compounds, displaying a DPPH scavenging activity stronger than that of the roots and leaves (Fernandes et al., 2007).

The objectives of this study were to define and compare the phenolics and organic acids composition and the biological potentials of the inflorescence of three *Brassica* varieties: *B. oleracea* var. *costata*, *B. oleracea* var. *acephala* and *B. rapa* var. *rapa*. For these purposes, the phenolic profile was established by reversed-phase HPLC–DAD analysis, while organic acids were determined by HPLC–UV. The antioxidant capacity was assessed by scavenging assays against DPPH radical and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid). The antimicrobial potential was checked for three Gram-positive (*Bacillus cereus*, *B. subtilis* and *Staphylococus aureus*) and three Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) and two fungi species (*Candida albicans* and *Cryptococcus neoformans*).

2. Materials and methods

2.1. Standards and reagents

Malic, shikimic, fumaric, caffeic, *p*-coumaric acids were purchased from Sigma (St. Louis, MO, USA). Aconitic, citric, pyruvic, ferulic and sinapic acids, kaempferol 3-*O*-rutinoside and isorhamnetin 3-*O*-glucoside were from Extrasynthése (Genay, France). Methanol, formic and acetic acids were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA). DPPH, xanthine, xanthine oxidase (XO) grade I from buttermilk (EC 1.1.3.22), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), ferric chloride anhydrous ($FeCl_3$), ethylenediaminetetraacetic acid disodium salt (EDTA), ascorbic acid, trichloroacetic acid, thiobarbituric acid, deoxyribose, sodium hypochlorite solution with 4% available chlorine (NaOCl), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. (St. Louis, USA).

2.2. Samples

Inflorescences of *B. oleracea* L. var. *costata* DC, *B. oleracea* L. var. *acephala* and *B. rapa* L. var. *rapa* were collected in Carrazeda de Ansiães, Northeast Portugal, in February 2006. After harvesting, the material of three distinct individuals of each variety was immediately transferred to the laboratory and frozen at −20 °C, prior to their lyophilisation in a Labconco 4.5 Freezone apparatus (Kansas City, MO, USA). Then the dried material was powdered, mixed and stored in a desiccator, in the dark.

2.3. Sample preparation

An aqueous extract was used for the phytochemical characterization and in the biological activities assays: ca. 3.0 g of powdered inflorescences were boiled for 15 min in 600 ml water and then filtered over a Büchner funnel. The resulting extract was lyophilized in a Labconco 4.5 Freezone apparatus (Kansas City, MO, USA) and yields of ca. 1.2 g (*B. oleracea* L. var. *costata* DC), 1.2 g (*B. rapa* L. var. *rapa*), and 0.8 g (*B. oleracea* L. var. *acephala*) were obtained. The lyophilized extracts were kept in a desiccator, in the dark.

For the characterization and quantification of the phenolic compounds by HPLC–DAD, each lyophilized extract was redissolved in water. For organic acids determination they were redissolved in sulphuric acid 0.01 N prior to analysis by HPLC–UV.

2.4. HPLC–DAD analysis of phenolic compounds

Twenty microliters of inflorescences lyophilized extracts were analyzed using a HPLC unit (Gilson) and a Spherisorb ODS2 (25.0 × 0.46 cm; 5 μm, particle size) column. The *B. oleracea* varieties (*costata* and *acephala*) were analyzed as previously described (Ferreres et al., 2005), using a mixture of formic acid 5% (A) and methanol (B), with a flow rate of 1 ml/min, as follows: 0 min – 10% B, 25 min – 20% B, 40 min – 50% B, 45 min – 50% B, 46 min – 90% B, 50 min – 90% B, 55 min – 100% B, 58 min – 100% B, 60 min – 10% B.

The separation of *B. rapa* var. *rapa* phenolic compounds was achieved as before (Fernandes et al., 2007), with a solvent mixture of water (adjusted to pH 3.2 with formic acid at 10%, v/v) (A) and methanol (B). Elution was carried out at 1 ml/min and followed the gradient system 20% B at 0 min, 50% B at 35 min, 80% B at 45 min and 100% B at 50 min. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200–400 nm, and chromatograms were recorded at 330 nm. The data were processed on Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Since standards of several compounds identified in the lyophilized extracts were not commercially available, 3-*p*-coumaroylquinic acid was quantified as *p*-coumaric acid, and sinapic acid, kaempferol and isorhamnetin derivatives as sinapic acid, kaempferol 3-*O*-rutinoside and isorhamnetin 3-*O*-glucoside, respectively. The other compounds were quantified as themselves.

2.5. HPLC–UV analysis of organic acids

The separation of the organic acids present in the inflorescences lyophilized extracts was carried out as previously reported (Sousa et al., 2005), in a system consisting of an analytical HPLC unit (Gilson) with an ion exclusion column, Nucleogel® Ion 300 OA (300 × 7.7 mm) in conjunction with a column heating device set at 30 °C. Briefly, elution was carried out isocratically, at a solvent flow rate of 0.2 ml/min, with sulphuric acid 0.01 N. The detection was performed with an UV detector set at 214 nm.

Organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards.

2.6. DPPH· scavenging activity

The antiradical activity of the extracts was determined spectrophotometrically in a Multiscan Ascent plate reader (Thermo Electron Corporation), by monitoring the disappearance of DPPH· at 515 nm, according to a described procedure (Ferreres et al., 2006; Vrchovská et al., 2006). For each extract, a dilution series composed of five different concentrations was prepared in a 96 well plate. The reaction mixtures in the sample wells consisted of 25 μl aqueous extract and 200 μl of 150 μM DPPH· dissolved in methanol. The plate was incubated for 30 min at room temperature. Three experiments were performed in triplicate.

2.7. Superoxide radical-scavenging activity

Antiradical activity of the aqueous extracts was determined spectrophotometrically in a Multiscan Ascent plate reader (Thermo Electron Corporation), by monitoring at 562 nm the formation of formazan as a result of the superoxide radical-induced reduction of NBT.

2.7.1. Non-enzymatic assay

Superoxide radicals were generated by the NADH/PMS system according to a described procedure (Valentão et al., 2001). All components were dissolved in phosphate buffer (19 mM, pH 7.4). Experiments were performed in triplicate.

2.7.2. Enzymatic assay

Superoxide radicals were generated by the xanthine/xanthine oxidase (X/XO) system following as reported before (Valentão et al., 2001). Briefly, xanthine was dissolved in NaOH (1 μM) and subsequently in phosphate buffer (50 mM) with EDTA (0.1 mM, pH 7.8), xanthine oxidase in EDTA (0.1 mM) and the remaining components in phosphate buffer (50 mM) with EDTA (0.1 mM, pH 7.8). Experiments were performed in triplicate.

2.7.3. Effect on xanthine oxidase activity

The effect of the lyophilized extracts on xanthine oxidase activity was evaluated by measuring the formation of uric acid from xanthine in a double beam spectrophotometer (Heλios α, Unicam), at room temperature, according to a described procedure (Valentão et al., 2001). The reaction mixtures contained the same proportion of components as in the enzymatic assay for superoxide radical-scavenging activity, except NBT, in a final volume of 750 μl. The absorbance was measured at 295 nm for 2 min. Experiments were performed in triplicate.

2.8. Hydroxyl radical assay

The deoxyribose method for determining the scavenging effect of the aqueous extracts on hydroxyl radicals was performed as previously described (Valentão et al., 2002) in a double beam spectrophotometer (Heλios α, Unicam). Reaction mixtures contained 50 μM ascorbic acid, 40 μM $FeCl_3$, 2 mM EDTA, 2.8 mM H_2O_2, 2.8 mM deoxyribose and lyophilized extracts. All components were dissolved in KH_2PO_4–KOH buffer 10 mM, pH 7.4. This assay was also performed either without ascorbic acid or EDTA, in order to evaluate the extracts pro-oxidant and metal chelation potential, respectively. Experiments were performed in triplicate.

2.9. Hypochlorous acid scavenging activity

The inhibition of hypochlorous acid-induced 5-thio-2-nitrobenzoic acid (TNB) oxidation to 5,5′-dithiobis(2-nitrobenzoic acid) was performed according to a described procedure (Valentão et al., 2002), in a double beam spectrophotometer (Heλios α, Unicam). Hypochlorous acid and TNB were prepared immediately before use. Experiments were performed in triplicate.

2.10. Antimicrobial activity

2.10.1. Microorganisms and culture conditions

Microorganisms CECT were obtained from the Spanish type culture collection (CECT) of Valencia University, while microorganisms ESA were clinically isolated strains identified in the Microbiology Laboratory of Escola Superior Agrária de Bragança. Gram-positive (*B. cereus* CECT 148, *B. subtilis* CECT 498 and *S. aureus* ESA 7 isolated from pus) and Gram-negative (*E. coli* CECT 101, *P. aeruginosa* CECT 108 and *K. pneumoniae* ESA 8 isolated from urine) bacteria, and fungi (*C. albicans* CECT 1394 and *C. neoformans* ESA 3 isolated from vaginal fluid) were used to screen the antimicrobial potential of the three *Brassica* varieties. Microorganisms were cultured aerobically at 37 °C (Scientific 222 oven) in nutrient agar medium for bacteria, and at 30 °C (Scientific 222 oven) in Sabouraud dextrose agar medium for fungi.

2.10.2. Assay

The screening of antibacterial activities against Gram-positive and Gram-negative bacteria and fungi and the determination of the minimal inhibitory concentration (MIC) were achieved by an adaptation of the agar streak dilution method based on radial diffusion, as previously reported (Hawkey & Lewis, 1994; Pereira et al., 2006; Sousa et al., 2006). Suspensions of the microorganism were prepared to contain approximately 108 cfu/ml, and the plates containing agar medium were inoculated (100 μl; spread on the surface). Each sample (50 μl) was placed in a hole (3 mm depth, 4 mm diameter) made in the centre of the agar. The MIC was considered to be the lowest concentration of the tested sample able to inhibit the growth of bacteria or fungi, after 24 and 48 h, respectively. The diameters of the inhibition zones corresponding to the MICs were measured using a ruler, with an accuracy of 0.5 mm. Each inhibition zone diameter was measured three times (three different plates) and the average was considered. A control using only inoculation was also carried out.

3. Results and discussion

3.1. Phenolic composition of the inflorescences

The HPLC–DAD analysis allowed the identification of fourteen phenolic compounds in the inflorescences of *B. oleracea* var. *costata*: 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside, kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside, kaempferol 3-*O*-sophoroside-7-*O*-sophoroside, kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside, kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside, kaempferol 3-*O*-(sinapoyl)-sophoroside, kaempferol 3-*O*-(feruloyl)-sophorotrioside, kaempferol 3-*O*-(feruloyl)-sophoroside and kaempferol 3-*O*-sophoroside (Fig. 1A). All these compounds have been previously described in *B. oleracea* var. *costata* leaves (Ferreres et al., 2006, 2005; Sousa et al., 2005).

The same compounds were found in *B. oleracea* var. *acephala* inflorescences aqueous lyophilized extract (Fig. 1B), with the exception of kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside (compound 7). Among the detected phenolics, only kaempferol 3-*O*-sophoroside-7-*O*-glucoside has been reported in the leaves of this *B. oleracea* variety (Romani et al., 2003).

B. rapa var. *rapa* inflorescences exhibited several phenolic acids and flavonoids distinct from those found in the *B. oleracea* varieties, namely isorhamnetin derivatives. Besides 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside-7-*O*-sophoroside, kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside and kaempferol 3-*O*-sophoroside detected in the above mentioned varieties, also identified were caffeic, ferulic and sinapic acids, kaempferol 3,7-*O*-diglucoside, isorhamnetin 3,7-*O*-diglucoside, 1,2-disinapoylgentiobiose, 1,2′-disinapoyl-2-feruloylgentiobiose, kaempferol 3-*O*-glucoside and isorhamnetin 3-*O*-glucoside (Fig. 2). These compounds have been already described in *B. rapa* var. *rapa* leaves and inflorescences (Fernandes et al., 2007). In addition, as observed before with other materials, isorhamnetin derivatives are present in *B. rapa* group and absent in *B. oleracea* (Romani et al., 2006).

The quantification of the identified phenolics in the three analyzed *Brassica* varieties inflorescences revealed that *B. oleracea* var. *costata* and *B. rapa* var. *rapa* present the highest contents (ca. 20 and 18 g/kg, respectively), corresponding to twice more the amount exhibited by *B. oleracea* var. *acephala* (ca. 9 g/kg) (Tables 1 and 2).

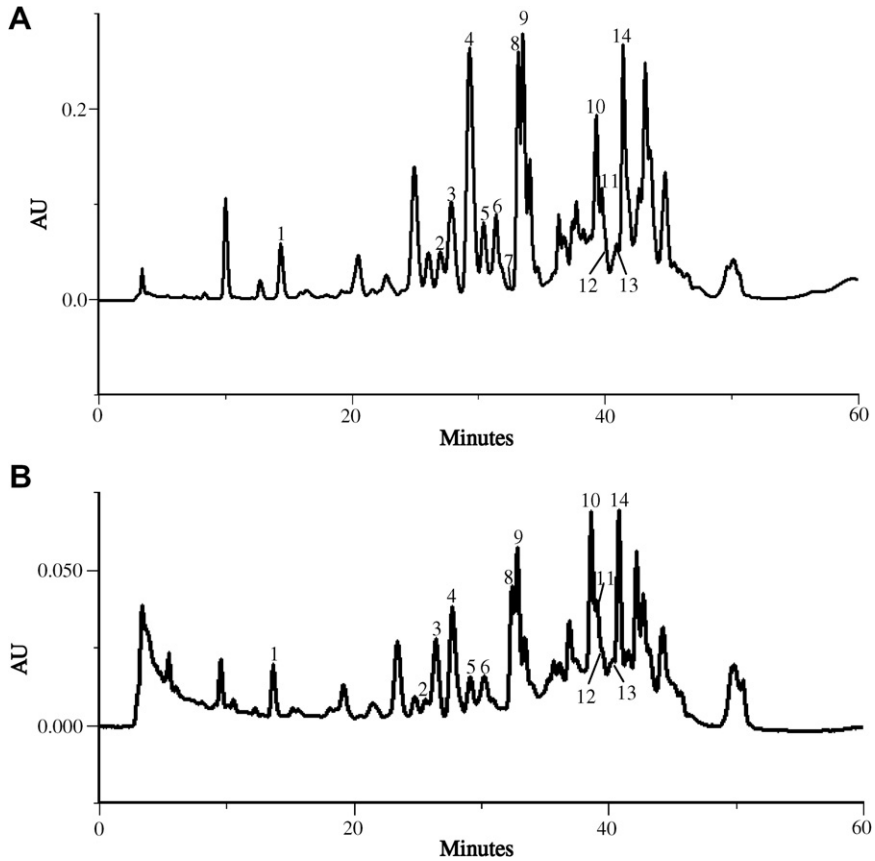

Fig. 1. HPLC–DAD phenolics profile of (A) *Brassica oleracea* var. *costata* and (B) *Brassica oleracea* var. *acephala* inflorescences aqueous lyophilized extracts. Detection at 330 nm. Peaks: (1) 3-*p*-coumaroylquinic acid; (2) kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside; (3) kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside; (4) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (5) kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside; (6) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside; (7) kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside; (8) kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside; (9) kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside; (10) kaempferol 3-*O*-sophorotrioside; (11) kaempferol 3-*O*-(sinapoyl)-sophoroside; (12) kaempferol 3-*O*-(feruloyl)-sophorotrioside; (13) kaempferol 3-*O*-(feruloyl)-sophoroside and (14) kaempferol 3-*O*-sophoroside.

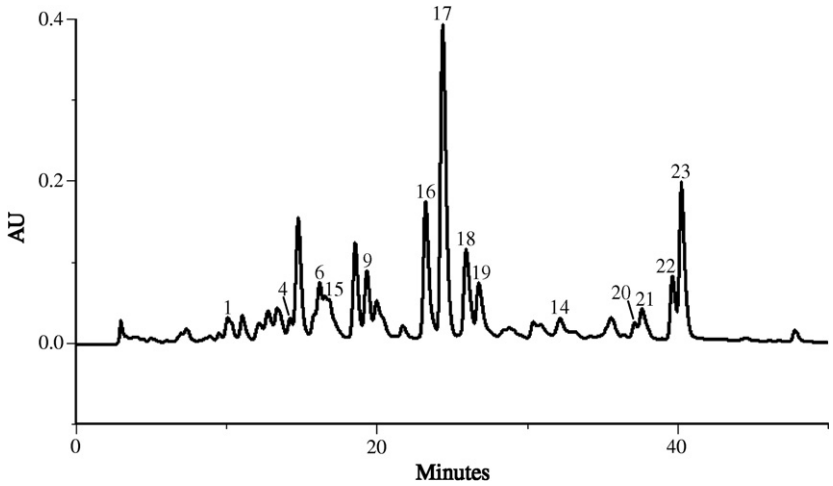

Fig. 2. HPLC–DAD phenolics profile of *B. rapa* var. *rapa* inflorescences aqueous lyophilized extract. Detection at 330 nm. Peaks: (1) 3-*p*-coumaroylquinic acid; (4) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (6) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside (15) caffeic acid; (9) kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside; (16) kaempferol 3,7-*O*-diglucoside; (17) isorhamnetin 3,7-*O*-diglucoside; (18) ferulic acid; (19) sinapic acid; (14) kaempferol 3-*O*-sophoroside; (20) 1,2-disinapoylgentiobiose; (21) 1,2′-disinapoyl-2-feruloylgentiobiose; (22) kaempferol 3-*O*-glucoside and (23) isorhamnetin 3-*O*-glucoside.

Despite their similar qualitative composition, the two *B. oleracea* varieties showed distinct profiles. In *B. oleracea* var. *costata* inflorescences kaempferol 3-*O*-sophoroside-7-*O*-glucoside is the main compound (corresponding to ca. 19% of total phenolics) and kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside the minor one (less than 0.5%), while in *B. oleracea* var. *acephala* kaempferol 3-*O*-sophoroside is the compound present in highest amounts (representing ca. 21% of total phenolics) and 3-*p*-coumaroylquinic acid

Table 1
Quantification of phenolic compounds in *Brassica oleracea* varieties' inflorescences

	Compound	mg/kg (dry basis)[a]	
		var. *costata*	var. *acephala*
1	3- *p*-Coumaroyl quinic acid	305.1 ± 2.9	165.6 ± 2.5
2	Kaempferol 3-*O*-sophtr-7-*O*-gluc	630.8 ± 4.4	172.8 ± 9.5
3	Kaempferol 3-*O*- (methoxycaffeoyl/caffeoyl)-soph-7-*O*-gluc	1508.7 ± 4.9	783.2 ± 32.0
4	Kaempferol 3-*O*-soph-7-*O*-gluc	3678.1 ± 10.0	839.5 ± 37.4
5	Kaempferol 3-*O*-sophtr-7-*O*-soph	989.5 ± 5.3	308.7 ± 3.7
6	Kaempferol 3-*O*-soph-7-*O*-soph	1263.2 ± 3.5	426.8 ± 12.5
7	Kaempferol 3-*O*-tetragluc-7-*O*-soph	78.4 ± 1.2	nd
8	Kaempferol 3-*O*-(sinapoyl/caffeoyl)-soph-7-*O*-gluc	1809.0 ± 40.3	663.9 ± 67.0
9	Kaempferol 3-*O*- (feruloyl/caffeoyl)-soph-7-*O*-gluc	2322.1 ± 19.1	1055.5 ± 66.7
10	Kaempferol 3-*O*-sophtr	1783.8 ± 9.9	1353.5 ± 18.0
11	Kaempferol 3-*O*-(sinapoyl)-soph	1345.3 ± 3.8	1148.9 ± 58.4
12	Kaempferol 3-*O*-(feruloyl)-sophtr		
13	Kaempferol 3-*O*-(feruloyl)-soph	760.2 ± 1.1	533.1 ± 42.0
14	Kaempferol 3-*O*-soph	3093.6 ± 3.5	2003.9 ± 121.6
	∑	19567.7	9455.4

[a] Results are expressed as mean ± standard deviation of three determinations. ∑, sum of the determined phenolic compounds. nd: not detected. sophtr: sophorotriose; soph: sophorose; gluc: glucose.

Table 2
Quantification of phenolic compounds in *Brassica rapa* var. rapa inflorescences

	Compound	mg/kg (dry basis)[a]
1	3-*p*-Coumaroyl quinic acid	1084.8 ± 10.5
4	Kaempferol 3-*O*-soph-7-*O*-gluc	479.8 ± 24.4
6	Kaempferol 3-*O*-soph-7-*O*-soph	2098.5 ± 82.7
15	Caffeic acid	422.2 ± 10.5
9	Kaempferol 3-*O*-(feruloyl/caffeoyl)-soph-7-*O*-gluc	2109.7 ± 165.4
16	Kaempferol 3,7-*O*-digluc	1208.9 ± 94.8
17	Isorhamnetin 3,7-*O*-digluc	3483.8 ± 23.1
18	Ferulic acid	2189.8 ± 58.3
19	Sinapic acid	790.4 ± 7.8
14	Kaempferol 3-*O*-soph	2127.8 ± 97.9
20	1,2-Disinapoyl-gentiobiose	136.3 ± 14.1
21	1,2′-Disinapoyl-2-feruloyl-gentiobiose	103.3 ± 2.3
22	Kaempferol 3-*O*-gluc	734.0 ± 18.7
23	Isorhamnetin 3-*O*-gluc	1414.3 ± 8.3
	∑	18383.8

[a] Results are expressed as mean ± standard deviation of three determinations. ∑, sum of the determined phenolic compounds; nd: not detected; soph: sophorose and gluc: glucose.

and kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside are the less abundant, each one corresponding to ca. 2%. In these varieties the phenolic acids contribution is very small, ca. 2% of total phenolics in each variety, being clearly distinct from the ca. 26% exhibited by the inflorescences of *B. rapa* var. *rapa* (Tables 1 and 2). In this latter species, isorhamnetin 3,7-*O*-diglucoside is the major compound, accounting for 19% of total phenolics, and 1,2′-disinapoyl-2-feruloylgentiobiose is the one present in lowest amounts (ca. 1%).

3.2. Organic acids in the inflorescences

An identical qualitative profile was found for the three analyzed *Brassica* varieties, which was composed by six organic acids: aconitic, citric, pyruvic, malic, shikimic and fumaric acids (Fig. 3). All these compounds were already described to occur in both *B. oleracea* var. *costata* (Ferreres et al., 2007, 2006; Sousa et al., 2005) and *B. rapa* var. *rapa* (Fernandes et al., 2007), with the exception of pyruvic acid that is identified for the first time in these varieties. Additionally, ascorbic acid that was present in leaves and seeds of *B. oleracea* var. *costata* (Ferreres et al., 2007, 2006; Sousa et al., 2005) was not detected in its inflorescences. Regarding *B. oleracea* var. *acephala*, only citric and malic acids were previously reported in the leaves (Ayaz et al., 2006).

From a quantitative point of view, *B. oleracea* var. *acephala* inflorescences showed the highest organic acids content (ca. 163 g/kg), corresponding to about three and four times the amount found for those of *costata* variety and *B. rapa* var. *rapa*, respectively (Table 3). *B. oleracea* var. *costata* exhibited a profile in which citric acid was the main compound (ca. 57% of total organic acids) and shikimic acid the minor one (less than 0.5%). In *B. oleracea* var. *acephala* and *B. rapa* var. *rapa* malic acid was the major organic acid, corre-

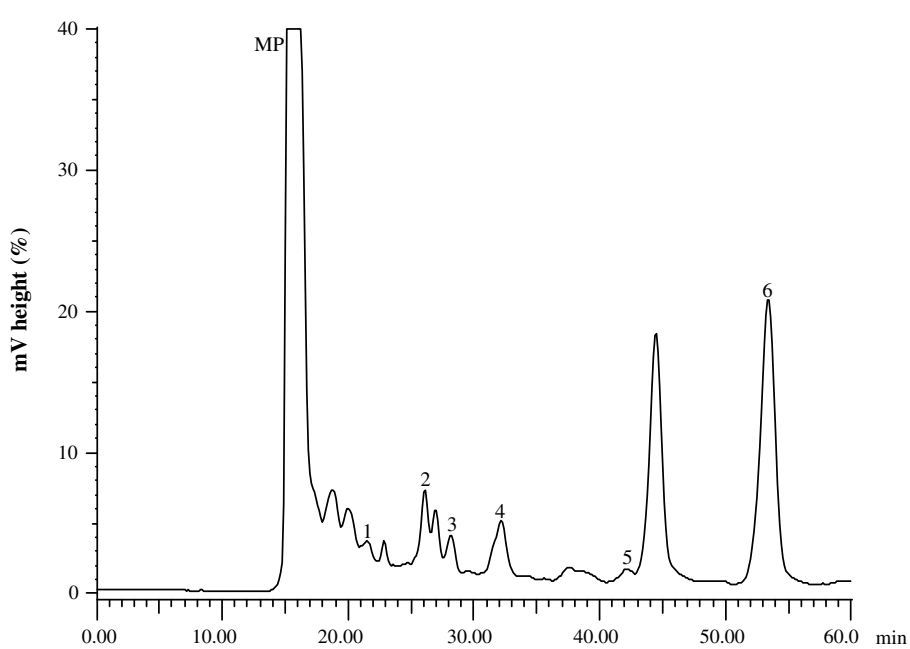

Fig. 3. HPLC–UV organic acid profile of *Brassica rapa* var. *rapa* inflorescences aqueous lyophilized extract. Detection at 214 nm. Peaks: (MP) mobile phase; (1) aconitic acid; (2) citric acid; (3) pyruvic acid; (4) malic acid; (5) shikimic acid and (6) fumaric acid.

Table 3
Quantification of organic acids in *Brassica* inflorescences (mg/kg, dry basis)[a]

Compound	*B. oleracea* var. *costata*	*B. oleracea* var. *acephala*	*B. rapa* var. *rapa*
Aconitic acid	426.7 ± 7.7	97.0 ± 2.6	42.3 ± 0.2
Citric acid	27925.7 ± 166.9	48373.3 ± 1846.1	13177.3 ± 75.4
Pyruvic acid	2684.0 ± 5.6	5686.7 ± 77.3	1123.2 ± 1.0
Malic acid	16734.2 ± 78.9	108158.7 ± 445.9	22349.6 ± 2.1
Shikimic acid	137.4 ± 0.6	764.7 ± 6.4	68.7 ± 0.8
Fumaric acid	1115.9 ± 4.4	17.5 ± 0.3	1260.8 ± 2.0
∑	49023.9	163097.9	38022.0

[a] Results are expressed as mean ± standard deviation of three determinations. ∑, sum of the determined organic acids.

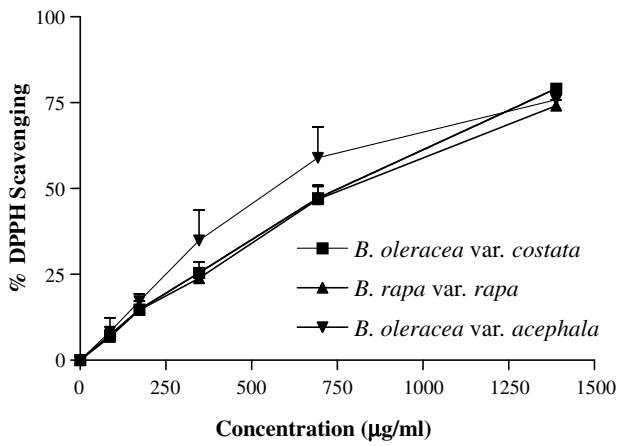

Fig. 4. Effect of inflorescences aqueous lyophilized extracts on DPPH· reduction. Values show mean ± SE from three experiments performed in triplicate.

Table 4
Antioxidant activity of the inflorescences' aqueous extracts (μg/ml)

Assay	*B. oleracea* var. *costata*	*B. oleracea* var. *acephala*	*B. rapa* var. *rapa*
DPPH[a]	754	565	774
Superoxide radical (X/XO)[b]	507	405	244
Superoxide radical (NADH/PMS)[b]	349	281	363
Hydroxyl radical[b]	172	10	12
HOCl[c]	639	1186	770

[a] Data correspond to IC_{50} values.
[b] Data correspond to IC_{25} values.
[c] Data correspond to IC_{10} values.

sponding to ca. 66 and 59% of total compounds, respectively. In both cases aconitic acid was the compound present in lowest amount, representing ca. 0.1% of total acids. Despite this coincidence, it can be noticed that, comparing with *B. oleracea* var. *acephala*, *B. rapa* var. *rapa* has a higher relative content of citric acid. On the other hand, its malic acid amount is inferior to that of *B. oleracea* var. *acephala*. Nevertheless, and according to the obtained results, it is evident that both citric and malic acids are the most important compounds: the sum of their amounts in the three analyzed *Brassica* inflorescences varies between ca. 91% and 94% of total organic acids (Table 3).

3.3. Antioxidant activity

The *Brassica* inflorescences were screened by the DPPH· assay, which provides basic information about their capacity to scavenge free radicals. In this assay the three varieties displayed a concentration dependent antioxidant potential. *B. oleracea* var. *acephala* was revealed to have a stronger capacity than *B. oleracea* var. *costata* and *B. rapa* var. *rapa*, which exhibited a similar behavior (Fig. 4, Table 4).

The three varieties exhibited a concentration dependent superoxide radical-scavenging activity, using the enzymatic system, and *B. rapa* var. *rapa* was the most effective one (Fig. 5A, Table 4). The effect of the aqueous lyophilized extracts on XO activity was also checked; once in this assay the inhibitory effect on the enzyme itself could also lead to a decrease of NBT reduction (Valentão et al., 2001). Thus, a control experiment monitoring the metabolic conversion of xanthine to uric acid was performed, revealing that for

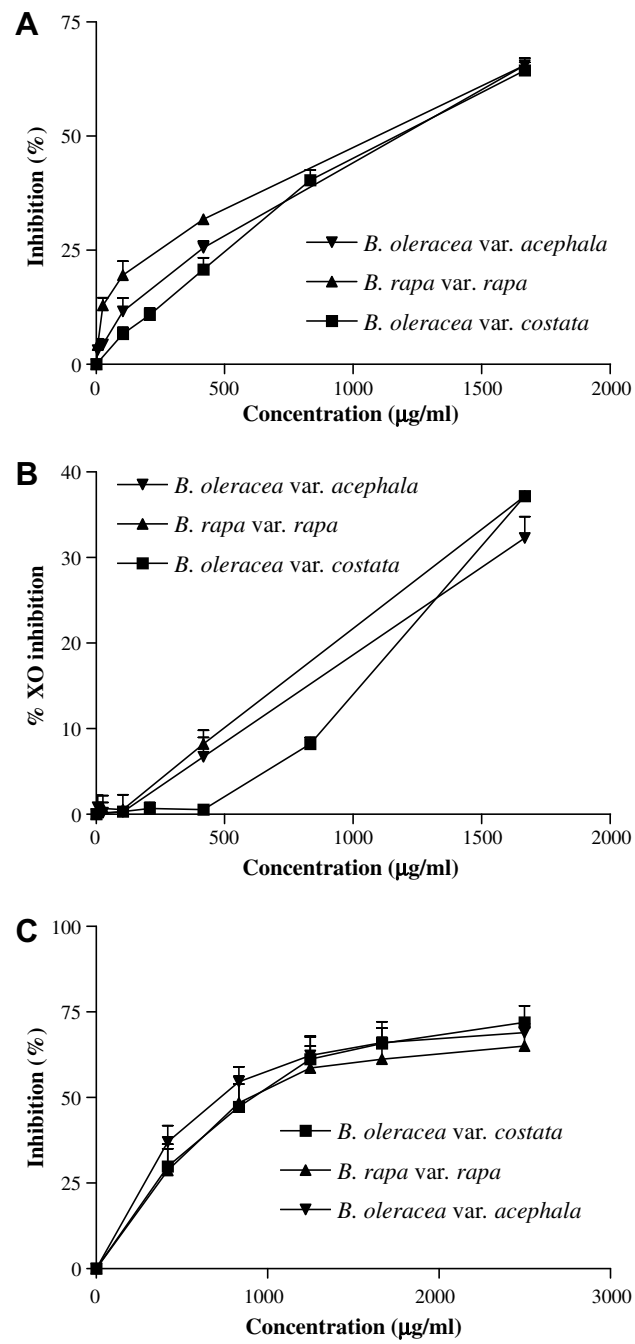

Fig. 5. Effect of inflorescences aqueous lyophilized extracts against superoxide radical generated in X/XO system (A), on XO activity (B), and against superoxide radical generated in NADH/PMS system (C). Values show mean ± SE from three experiments performed in triplicate.

concentrations above 104 μg/ml both *B. oleracea* var. *acephala* and *B. rapa* var. *rapa* have XO inhibitory capacity. *B. oleracea* var. *costata* was also able to inhibit this enzyme, but only for concentrations higher than 417 μg/ml (Fig. 5B). Considering these results it was not possible to show a clear-cut scavenging effect on superoxide radical. To confirm the scavenging capacity we also determined the effect of the extracts on superoxide radical generated in a chemical system, and a concentration dependent effect was observed, with *B. oleracea* var. *acephala* displaying the stronger capacity (Fig. 5C, Table 4).

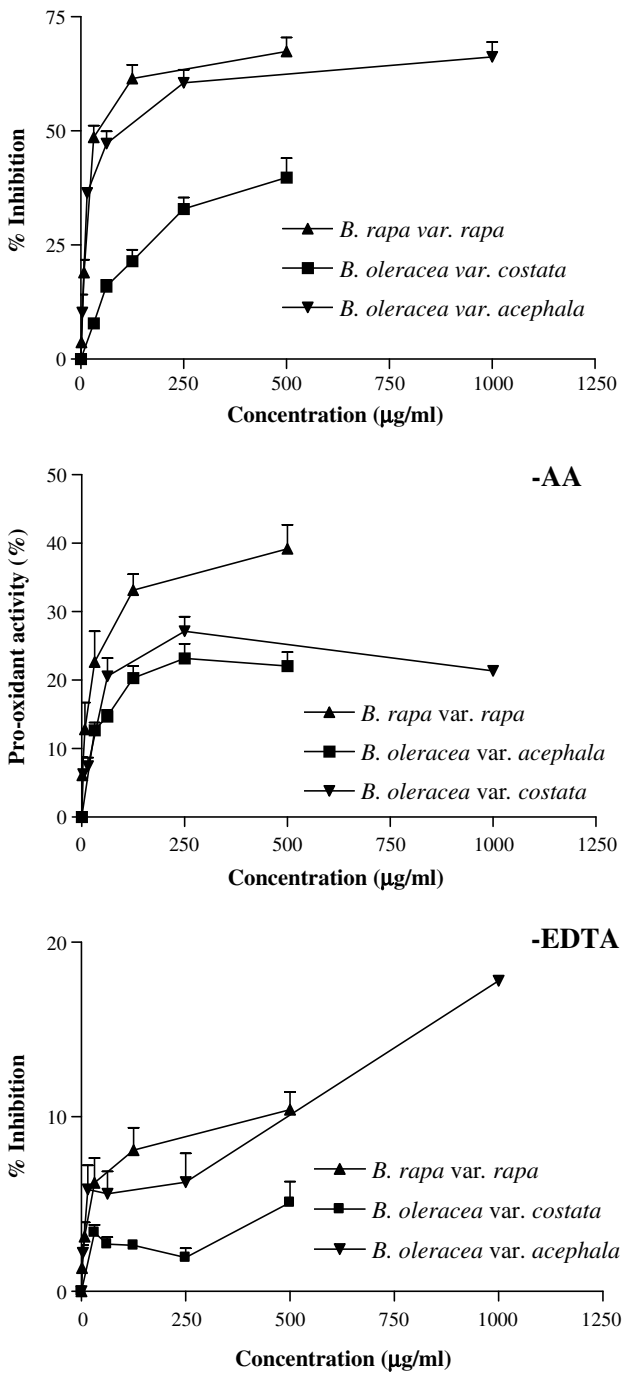

Fig. 6. Inflorescences aqueous lyophilized extracts non-specific hydroxyl radical-scavenging activity, pro-oxidant activity (-AA) and specific hydroxyl radical-scavenging (-EDTA). Values show mean ± SE from three experiments performed in triplicate.

B. oleracea var. *acephala* and *B. rapa* var. *rapa* lyophilized extracts also exhibited a similar potent scavenging activity for hydroxyl radical, in a concentration dependent manner, which was more pronounced than that of *B. oleracea* var. *costata* (Fig. 6, Table 4). If we omit ascorbate from the reaction mixture, and if pro-oxidant compounds are present, they will be able to redox cycle the metal ion required for hydroxyl generation, thus increasing the radical production (Valentão et al., 2002). In order to evaluate the pro-oxidant potential of the three inflorescences, we omitted ascorbic acid, and we found that they were effective substitutes for ascorbate, although *B. oleracea* var. *costata* presented pro-oxidant capacity only for concentrations below 250 μg/ml (Fig. 6). So, it seems that, at the tested concentrations, the three inflorescences have both anti-oxidant and pro-oxidant effects, with the first being more pronounced than the latter. Some compounds prevent deoxyribose damage in this assay, not by reacting with hydroxyl radicals, but because they present ion-binding capacity and can withdraw the iron ions rendering them inactive or poorly active in Fenton reactions (Valentão et al., 2002). The assay performed in the absence of EDTA showed that the three *Brassica* varieties have some capacity to chelate iron ions, being *B. oleracea* var. *acephala* the most effective (Fig. 6).

The oxidizing properties of HOCl induce the conversion of TNB to DTNB, which is inhibited by a HOCl scavenger (Valentão et al., 2002). The analyzed inflorescences displayed protective activity against damage by HOCl, which was concentration dependent. Among the distinct varieties *B. oleracea* var. *costata* and *B. rapa* var. *rapa* revealed to have higher scavenging ability, as shown in Fig. 7 and Table 4.

3.4. Antimicrobial activity

The aqueous extracts of the inflorescences were screened for their antimicrobial properties against *B. cereus*, *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *C. albicans* and *C. neoformans*. The minimal inhibitory concentration (MIC) values found for the tested bacteria and fungi (Table 5) were determined as an evaluation of the antimicrobial activity of the samples.

All the extracts presented antimicrobial capacity, inhibiting only Gram-positive bacteria and in the order *S. aureus* > *B. cereus* >> *B. subtilis*. Despite this, the response of each *Brassica* variety against the assayed microorganisms was different. *B. rapa* var. *rapa* revealed the highest activity against *B. cereus*, followed by *B. oleracea* var. *costata* and *B. oleracea* var. *acephala*. Only *B. rapa* var. *rapa* and *B. oleracea* var. *costata* showed some activity against *B. subtilis*. *S. aureus* was the most susceptible microorganism, presenting

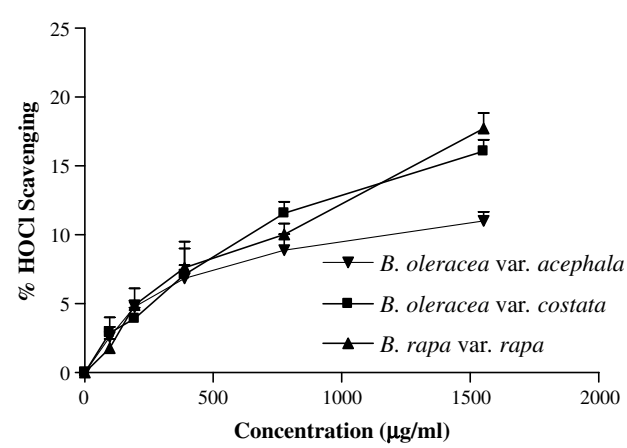

Fig. 7. Effect of inflorescences aqueous lyophilized extracts on the oxidation of TNB by HOCl. Values show mean ± SE from three experiments performed in triplicate.

Table 5
Antimicrobial activity of the inflorescences' aqueous extracts[a]

Samples	MIC (mg/ml)							
	B. cereus	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*K. peumoniae*	*C. albicans*	*C. neoformans*
B. rapa	0.1	10.0	0.1	50.0	50.0	50.0	50.0	50.0
var. *rapa*	(+ + +)	(+)	(+ + +)	(−)	(−)	(−)	(−)	(−)
B. oleracea	0.1	10.0	0.1	50.0	50.0	50.0	50.0	50.0
var. *costata*	(+ +)	(+)	(+ + + +)	(−)	(−)	(−)	(−)	(−)
B. oleracea	0.1	50.0	0.1	50.0	50.0	50.0	50.0	50.0
var. *acephala*	(+)	(−)	(+ + +)	(−)	(−)	(−)	(−)	(−)

[a] No antimicrobial activity (−), inhibition zone < 1 mm. Slight antimicrobial activity (+), inhibition zone 2–3 mm. Moderate antimicrobial activity (+ +), inhibition zone 4–5 mm. High antimicrobial activity (+ + +), inhibition zone 6–9 mm. Strong antimicrobial activity (+ + + +), inhibition zone > 9 mm. Standard deviation ± 0.5 mm.

MICs of 0.1 mg/ml for the three inflorescences tested, with *B. oleracea* var. *costata* displaying the best antimicrobial capacity (Table 5). The tested Gram-negative bacteria (*E. coli*, *P. aeruginosa* and *K. peumoniae*) and fungi (*C. albicans* and *C. neoformans*) species were resistant to the inflorescences extracts (Table 5).

The chemical composition of the analyzed extracts can obviously be very complex and may contain several classes of hydrophilic compounds, besides the phenolics and organic acids indicated above. Consequently, it seems important to evaluate the activity of the inflorescences aqueous lyophilized extracts as a whole, because interactions may occur among the different compounds present. Although no correlation was found between the phenolics or organic acids contents and the observed activities, the detected compounds are, most probably, contributing to them. In fact, hydroxycinnamic acids and their derivatives (Fukumoto & Mazza, 2000; Plumb, Price, Rhodes, & Williamson, 1997), flavonol glycosides, including acylated derivatives (Braca et al., 2003; Tang, Lou, Wang, Li, & Zhuang, 2001), or organic acids (Madhavi, Singhal, & Kulkarni, 1996; Silva et al., 2004) have been reported to possess antioxidative properties, assessed in different systems. Additionally, the antimicrobial capacity of these phytochemicals against several microorganisms was also demonstrated before (Alakomi et al., 2007; Binutu, Adesogan, & Okogun, 1996; Bloor, 1995; Lee, Thrupp, Owens, Cesario, & Shanbrom, 2001; Mokbel & Suganuma, 2006; Ou & Kwok, 2004; Pomilio, Buschi, Tomes, & Viale, 1992; Rigano et al., 2007). The obtained results are important, considering that the studied reactive oxygen species are produced in the organism or come from exogenous sources, being involved in several diseases (Aruoma, Halliwell, Hoey, & Butler, 1989; Bast, Haenen, & Doelman, 1991; Halliwell, 1991; Halliwell, Aeschbach, Löliger, & Aruoma, 1995; Puppo, 1992). Furthermore, the dietary intake of these inflorescences may lower the risk of bacterial infections, namely of the gastrointestinal tract, being also useful in food industry as preservative (Frazier & Westhoff, 1988).

In conclusion, the work herein indicates that the inflorescences of the three analyzed *Brassica* varieties are an appreciable source of protective compounds, like phenolics and organic acids. In addition, it points to the need of a diverse diet to get the most complete protection, through overlapping or complementary effects, as it is not possible to suggest one variety as being the best in terms of antioxidant or antimicrobial capacity.

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4.13. Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC): Scavenger of reactive nitrogen species

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Tronchuda Cabbage (*Brassica oleracea* L. var. *costata* DC): Scavenger of Reactive Nitrogen Species

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The ability of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) to act as a scavenger of the reactive nitrogen species nitric oxide and peroxynitrite was investigated. The aqueous extracts obtained from tronchuda cabbage seeds and from its external and internal leaves exhibited a concentration dependent scavenging capacity. The antioxidant potential observed against the two reactive species was as follows: seeds > external leaves > internal leaves. In order to establish a possible correlation with the chemical composition of the extracts, the activity of ascorbic and sinapic acids and kaempferol 3-*O*-rutinoside was also studied. Among the compounds tested, sinapic acid showed the strongest antioxidant activity against both species.

KEYWORDS: ***Brassica oleracea* L. var. *costata* DC; Brassicaceae; nitric oxide; peroxynitrite; phenolic compounds; ascorbic acid**

INTRODUCTION

Nitric oxide (NO) is an important molecule involved in several physiological processes, which include blood pressure control, neural signal transduction, platelet function, and antimicrobial defense, among others (*1–4*). NO is produced in various cell types by nitric oxide synthases and reacts rapidly with superoxide to form peroxynitrite ($ONOO^-$), essential in the defensive process against invading microorganisms *in vivo* (*5*).

Despite their beneficial effects, an overproduction of these reactive nitrogen species (RNS) is associated with several types of biological damage. Deleterious effects include lipid peroxidation, protein oxidation and nitration, enzyme inactivation, and DNA damage (*6*), which lead to chronic inflammation, cardiovascular diseases, Parkinson's and Alzheimer's diseases, and several types of cancer (*7, 8*). Scavengers of RNS, especially those from exogenous sources, may play a pivotal role in preventing/controlling degenerative diseases. Antioxidants must react with reactive species faster than biological substrates, thus protecting biological targets from oxidative damage (*9*).

With this respect, it has been shown that fruits and vegetables can be a constant source of health-promoting compounds (*8, 10*). One group of vegetables that has been regarded as potentially cancer protective is the Brassicaceae family. The consumption of *Brassica* vegetables throughout the world is enormous and they constitute an important part of a well balanced diet. They are reported to reduce the risks of some cancers especially due to its glucosinolates and their derived products (*11, 12*), although phenolic compounds are also found to contribute to this ability (*13*).

Among *Brassica* species, tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) is commonly consumed, being widely used in the preparation of soups or just boiled. Additionally, it also constitutes an ingredient of salads, particularly the internal leaves which are tender and sweeter than the external ones. Previous work on the chemical characterization of this species revealed distinct compositions of its external and internal leaves, seeds, and sprouts, in terms of phenolic compounds and organic acids (*14–19*). Tronchuda cabbage leaves and seeds were also shown to be effective scavengers of reactive oxygen species, namely superoxide and hydroxyl radicals and hypochloride (*14, 15, 18*), but, as far as we know, its activity against RNS has not been reported.

In this paper tronchuda cabbage aqueous extracts from internal and external leaves and from seeds were studied for their antioxidant ability against nitric oxide and peroxynitrite. With this purpose the capacity of tronchuda cabbage extracts to scavenge NO was evaluated by monitoring nitrite accumulation in the presence of nitroprusside. The protection against damage by $ONOO^-$ was assessed by their abilities to inhibit tyrosine nitration. In order to establish some correlation with the chemical composition of the extracts, phenolic compounds and organic acids were determined by HPLC-DAD and HPLC-UV, respectively, and the antioxidant capacity of ascorbic and sinapic acids and kaempferol 3-*O*-rutinoside was also evaluated.

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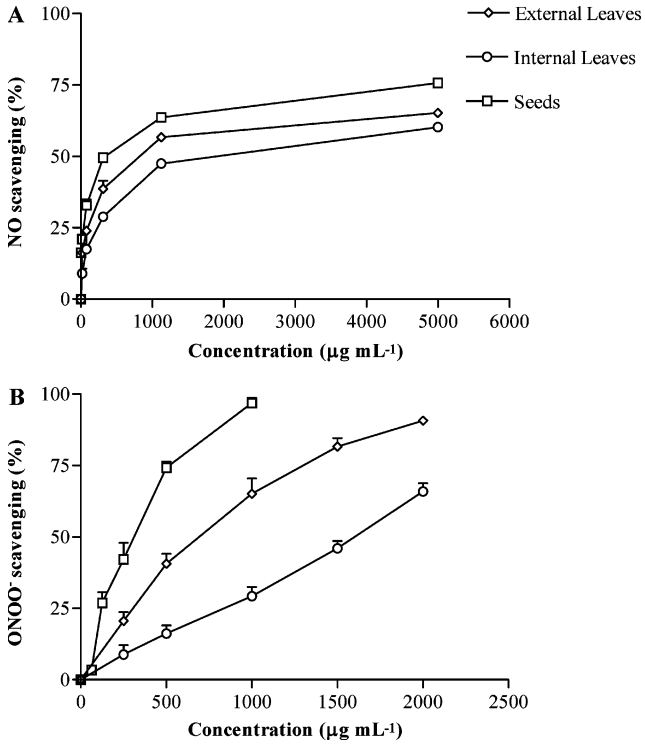

Figure 1. Effect of tronchuda cabbage extracts against (**A**) nitric oxide and (**B**) peroxynitrite. Values show mean + SE from three experiments, performed in triplicate.

Table 1. IC_{50} Values ± SE (μg mL^{-1}) of Tronchuda Cabbage against Reactive Nitrogen Species

sample	NO	ONOO
seeds	356 ± 58	302 ± 34
internal leaves	2228 ± 214	1641 ± 90
external leaves	884 ± 100	707 ± 92

MATERIALS AND METHODS

Standards and Reagents. Sinapic acid was from Sigma (St. Louis, MO), kaempferol 3-*O*-rutinoside and rutin from Extrasynthése (Genay, France), and L-(+)-ascorbic acid from Merck (Darmstadt, Germany). Methanol, hydrogen peroxide 30%, potassium dihydrogen phosphate, *N*-(1-naphthyl)ethylenediamine dihydrochloride, and phosphoric acid were obtained from Merck. Sodium nitroprussiate dihydrate was from Riedel-de Haën (St. Louis, MO), magnesium peroxide was from Aldrich (St. Louis, MO), DL-penicillamine, sulfanilamide, potassium nitrite, L-tyrosine, and 3-nitro-L-tyrosine were from Sigma. The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

Plant Material. Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) was produced under organic practices, as described previously (*15*). Three plants were randomly collected, and their external and internal leaves were separated, frozen, and lyophilized. External and internal leaf dried material was powdered and kept in a desiccator, in the dark. Tronchuda cabbage seeds were obtained from local farmers in Bragança, Northeast Portugal, which produce and commercialize tronchuda cabbage. Identification was performed by José A. Pereira, Ph.D. (CIMO/Escola Superior Agrária, Instituto Politécnico de Bragança). Voucher specimens (20041027E, 20041027I, and TCS) were deposited at Pharmacognosy Service, Faculty of Pharmacy, Porto University.

Extracts Preparation. Tronchuda cabbage leaf and seed extracts were prepared as previously reported (*14, 15*). Briefly, 3.0 g of lyophilized plant leaves or ground seeds were boiled for 1 h in 600 mL of water. The resulting extracts were filtered over a filtration funnel and then lyophilized in a Labconco FreeZone 4.5 apparatus. The lyophilized extracts were kept in a desiccator, in the dark. For phenolic compounds and organic acids analyses, they were redissolved in water and 0.01 N sulfuric acid, respectively.

Nitric Oxide Scavenging Activity. The antiradical activity was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc.), according to a described procedure (*20*), with modifications. A 100 μL amount of 20 mM sodium nitroprusside was incubated with 100 μL of sample (five different concentrations) for 60 min, at room temperature, under light. All solutions were prepared in 0.1 M phosphate buffer (pH 7.4). After incubation, 100 μL of Greiss reagent, containing 1% sulfanilamide and 0.1% naphthylethylenediamine in 2% phosphoric acid, was added to each well. The mixture was incubated at room temperature for 10 min, and the absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was read at 562 nm. Rutin was used as positive control (*21*).

Peroxynitrite Scavenging Activity. *Peroxynitrite Synthesis.* $ONOO^-$ was synthesized as previously reported (*22*). Briefly, 20 mL of an acidic solution (0.6 M HCl) of H_2O_2 (0.7 M) was mixed with 20 mL of KNO_2 (0.6 M), on ice, for 1 s, and the reaction was quenched with 20 mL of ice-cold NaOH (1.2 M). Residual H_2O_2 was removed by adding 10−15 mg of MnO_2. The solution was then filtered and frozen overnight at −20 °C. The yellow top layer of $ONOO^-$ formed by freeze fractionation was scraped, and the concentration of stock $ONOO^-$ was determined at 302 nm, using a molar extinction coefficient of 1670 cm^{-1} M^{-1}.

Nitration of Tyrosine by $ONOO^-$. For each extract, a dilution series (five different concentrations) and a control solution without sample was prepared. Tyrosine 10 mM was prepared by dissolving 18.1 mg of pure compound in 8 mL of water with 250 μL (10% w/v) of KOH, followed by neutralization with 250 μL of 5% phosphoric acid and addition of 1.5 mL of water. A 100 μL amount of tyrosine solution and 100 μL of sample were added to 795 μL of buffer (100 mM KH_2PO_4, pH 7.4) and incubated in a water bath at 37 °C, for 15 min. After this time, 5 μL of 200 mM $ONOO^-$ was added under vigorous stirring (15 s), and the resulting mixture (pH 7.4−7.5) was incubated at 37 °C for another15 min. DL-penicillamine was used as positive control (*23*).

HPLC Determination of 3-Nitrotyrosine. Measurement of 3-nitrotyrosine was performed with a HPLC unit (Gilson Medical Electronics, France), using a reversed phase Spherisorb ODS2 column (250 × 4.6 mm i.d., 5 μm; Waters, Milford, MA). The injection volume was 20 μL. The eluent was 500 mM KH_2PO_4−H_3PO_4, pH 3.0, with 20% methanol (v/v) at a flow rate of 0.6 mL min^{-1}. Detection was achieved with a Gilson UV detector set at 274 nm, and the peak areas were calculated using the 712 HPLC System Controller Software (Gilson).

HPLC-DAD Analysis of Phenolic Compounds. Twenty microliter amounts of lyophilized extracts were analyzed using an HPLC unit (Gilson) and a RP-18 LiChroCART (Merck, Darmstadt, Germany) column (250 × 4 mm, 5 μm particle size). Chromatographic separations of seed and internal leaf extracts were accomplished under the conditions described previously (*14, 15*), using acetic acid 1% (A) and methanol (B) as solvents. Elution was performed at a flow rate of 1 mL min^{-1}, starting with 20% B and using a gradient to obtain 50% B at 30 min and 80% B at 37 min.

For the external leaf extract, the solvent system was a mixture of formic acid 5% in water (A) and methanol (B), with a flow rate of 1 mL min^{-1}, and the gradient was as follows: 10% B at 0 min, 20% B at 25 min, 50% B at 40 min, 50% B at 45 min, 90% B at 46 min, 90% B at 50 min, 100% B at 55 min, 100% B at 58 min, and 10% B at 60 min (*15*).

In all cases detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200−400 nm, and chromatograms were recorded at 330 nm. The data were processed on a Unipoint Software system (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. As standards correspondent to the compounds identified in the lyophilized

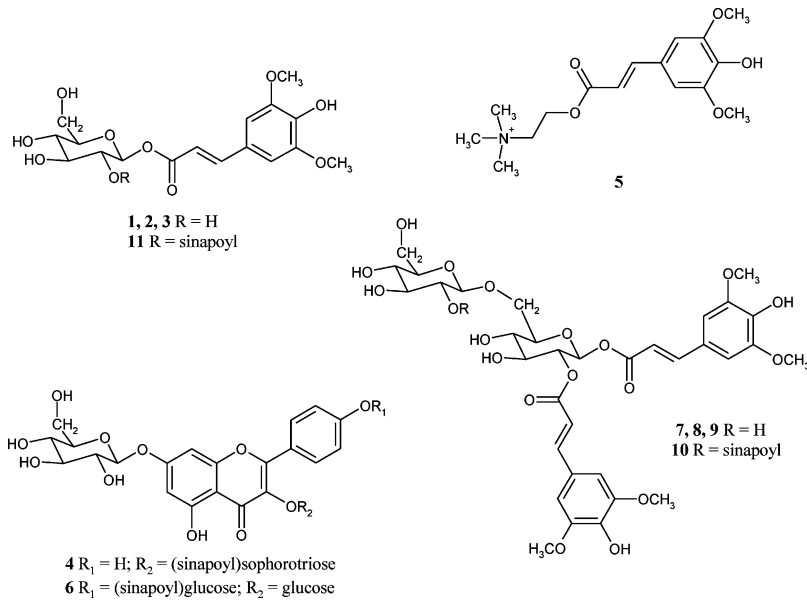

Figure 2. Chemical structures of several phenolic compounds present in tronchuda cabbage seeds. 1-Sinapoylglucose isomer (**1**); 1-sinapoylglucose isomer (**2**); 1-sinapoylglucose (**3**); kaempferol-3-*O*-(sinapoyl)sophorotrioside-7-*O*-glucoside (**4**); sinapoylcholine (**5**); kaempferol-3,7-*O*-diglucoside-4′-*O*-(sinapoyl)glucoside (**6**); 1,2-disinapoylgentiobiose isomer (**7**); 1,2-disinapoylgentiobiose isomer (**8**); 1,2-disinapoylgentiobiose (**9**); 1,2,2′-trisinapoylgentiobiose (**10**); 1,2-disinapoylglucose (**11**).

extracts were not commercially available, 3- and 4-*p*-coumaroylquinic acids were quantified as *p*-coumaric acid, sinapic acid derivatives as sinapic acid, and the kaempferol derivatives as kaempferol-3-*O*-rutinoside. Sinapic acid was quantified as itself.

HPLC-UV Analysis of Organic Acids. The separation of the organic acids present in the lyophilized extracts was carried out as previously reported (*14, 15*), in a system consisting of an analytical HPLC unit (Gilson) with an ion exclusion column, Nucleogel Ion 300 OA (300 × 7.7 mm), in conjunction with a column heating device set at 30 °C. Briefly, elution was carried out isocratically, at a solvent flow rate of 0.2 mL min^{-1}, with 0.01 N sulfuric acid. The detection was performed with a UV detector set at 214 nm. Organic acid quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Malic and quinic acids were quantified together as malic acid.

RESULTS AND DISCUSSION

RNS have several roles in mammals, but their unregulated production can cause adverse effects through reaction with biological target molecules. Our recent studies have demonstrated that tronchuda cabbage can provide protection against some reactive oxygen species (*14, 15, 18*). In the present work the study of the antioxidant capacity of tronchuda cabbage was extended to the RNS nitric oxide and peroxynitrite.

Nitric oxide is a short-lived free radical, which possesses a small dipole moment because of the similar electronegativity of oxygen and nitrogen, turning it essentially hydrophobic and freely diffusible across membranes (*24*). NO was generated from sodium nitroprusside, an inorganic complex used as NO donor: in aqueous solution at physiological pH, and under light irradiation, NO is spontaneously released from sodium nitroprusside (*25*). As NO is relatively unstable in the presence of molecular oxygen, it rapidly autoxidizes to yield a variety of nitrogen oxides, namely nitrogen dioxide, dinitrogen trioxide, and nitrite (*26*). Nitrite is the only stable product and can be estimated by use of Greiss reagent (*26*). In this assay scavengers of NO compete with oxygen, leading to reduced nitrite production (*20*). Tronchuda cabbage extracts showed a concentration-dependent protective effect against NO (**Figure 1A**), with the seed extract being the one with the major scavenging potential (**Table 1**). The external leaves were shown to have higher NO scavenging capacity than the internal ones (**Table 1**). Recently we have reported tronchuda cabbage antioxidant ability against superoxide radical (*14, 15, 18*). Now a scavenging activity towards nitric oxide was demonstrated, which may be important in preventing peroxynitrite formation by its reaction with superoxide radical.

Peroxynitrite is a major damaging oxidant produced *in vivo* that reacts slowly enough with most biological molecules to be partially selective in the types of molecules that it attacks (*27*). It is a potent nitrating agent that converts free and protein-bound tyrosine to the corresponding 3-nitro derivative (*28*), a stable adduct involving a carbon−nitrogen bond that is difficult to remove chemically (*27*). 3-Nitrotyrosine is considered to be a marker of $ONOO^-$-dependent damage *in vivo*: tyrosine nitration affects protein structure and function, contributing to further dysfunctional changes (*28, 29*). In this work 3-nitrotyrosine was formed from the reaction between free tyrosine and peroxynitrite, at physiological pH, and determined by HPLC-UV analysis. Thus, in the presence of peroxynitrite scavengers there will be an inhibition of tyrosine nitration. Qualitative analyses were previously performed to verify that there was no coelution with 3-nitrotyrosine. Tronchuda cabbage extracts effectively inhibited the formation of 3-nitrotyrosine, in a concentration dependent manner (**Figure 1B**). Seed extract displayed the most potent $ONOO^-$ scavenging capacity, followed by the external leaves (**Table 1**).

When comparing the results obtained here with those previously reported for reactive oxygen species (*14, 15, 18*), the same order of antioxidant capacity could be observed: tronchuda cabbage seeds exhibit the highest protective activity and the internal leaves the lowest one. The different results obtained with the three tested tronchuda cabbage extracts may be explained by their distinct chemical composition. In addition to the vitamins and minerals of fruits and vegetables, phytochemicals, such as phenolic compounds and organic acids, may contribute to their antioxidant capacity. The phenolic and organic acid composition of the tested extracts was determined by HPLC-DAD and HPLC-UV, respectively.

Table 2. Quantification of Phenolic Compounds (mg/kg, dry basis)[a]

compounds	seeds	internal leaves	external leaves
	Sinapic Acid Derivatives[b]		
SnGt	309 ± 0.3		
1-SnGl isomer	368 ± 11.7		
SnGt isomer	270 ± 1.7		
1-SnGl isomer	417 ± 9.8		
1-SnGl	703 ± 11.5		
SnCholine	376 ± 7.1		
1,2-diSnGt isomer	152 ± 3.2		
1,2-diSnGt isomer	345 ± 2.0		
1,2-diSnGt	1023 ± 37.1	52 ± 0.3	
1,2,2′-triSnGt	448 ± 2.2	63 ± 0.4	
1,2-diSnGl	368 ± 2.4		
SnGl acid		26 ± 0.2	
Sinapic acid		180 ± 1.1	
1,2′-diSn-2-FrGt		11 ± 0.1	
	p-Coumaric Acid Derivatives[c]		
3-*p*-CmQn		189 ± 1.4	482 ± 8.6
4-*p*-CmQn		126 ± 0.9	
	Kaempferol Derivatives[d]		
K-3-(Sn)Sphtri-7-Gl	911 ± 17.7		
K-3,7-diGl-4′-(Sn)Gl	267 ± 17.6		
K-3-Sph-7-Gl		288 ± 8.8	852 ± 14.0
K-3-(Cf)Sph-7-Gl		121 ± 3.7	
K-3-(Sn)Sph-7-Gl		181 ± 5.5	
K-3-(Fr)Sph-7-Gl		54 ± 1.6	
K-3-Sph		100 ± 3.1	797 ± 31.4
K-3-Sphtri-7-Gl + K-3-(MtCf/Cf)Sph-7-Gl			453 ± 12.3
K-3-Sphtri-7-Sph			445 ± 6.1
K-3-Sph-7-Sph			2788 ± 48.9
K-3-(Sn/Cf)Sph-7-Gl			1682 ± 21.2
K-3-(Fr/Cf)Sph-7-Gl			1886 ± 45.1
K-3-Sphtri			719 ± 7.2
K-3-(Sn)Sph			1244 ± 62.8
K-3-(Fr)Sphtri + K-3-(Fr)Sph			1989 ± 33.1
Σ	5974	1390	13337

[a] Results are expressed as mean ± standard deviation of three determinations. Σ, sum of the determined phenolic compounds. [b] Sinapic acid equivalents. [c] *p*-Coumaric acid equivalents. [d] Kaempferol-3-*O*-rutinoside equivalents. Cf, caffeoyl; Cm, coumaroyl; Fr, feruloyl; Gl, glucose; Gt, gentiobiose; K, kaempferol; Mt, methoxy; Qn, quinic; Sn, sinapoyl; Sph, sophorose; Sphtri, sophorotriose.

Tronchuda cabbage seed extract was characterized by the presence of two sinapoylgentiobiose isomers, three sinapoylglucose isomers, kaempferol-3-*O*-(sinapoyl)sophorotrioside-7-*O*-glucoside, sinapoylcholine, kaempferol-3,7-*O*-diglucoside-4′-*O*-(sinapoyl)glucoside, three disinapoylgentiobiose isomers, 1,2,2′-trisinapoylgentiobiose, and 1,2-disinapoylglucose (**Figure 2**). All these compounds were already reported to occur in this matrix (*14*). These phenolics were present in high content in the analyzed extract (ca. 6.0 g/kg), from which ca. 80% corresponded to the nonflavonoid sinapic acid derivatives (**Table 2**).

The external leaf extract exhibited 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside, kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside, kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside, kaempferol 3-*O*-sophoroside-7-*O*-sophoroside, kaempferol 3-*O*-(sinapoyl/caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside, kaempferol 3-*O*-(sinapoyl)sophoroside, kaempferol 3-*O*-(feruloyl)sophorotrioside, and kaempferol 3-*O*-(feruloyl)-sophoroside (**Figure 3**), which were already found previously in this material (*15, 16, 18*). This extract showed the highest phenolics amount (ca. 13 g/kg), in which the kaempferol derivatives were predominant, being *p*-coumaric acid derivative a minor compound (ca. 4%) (**Table 2**).

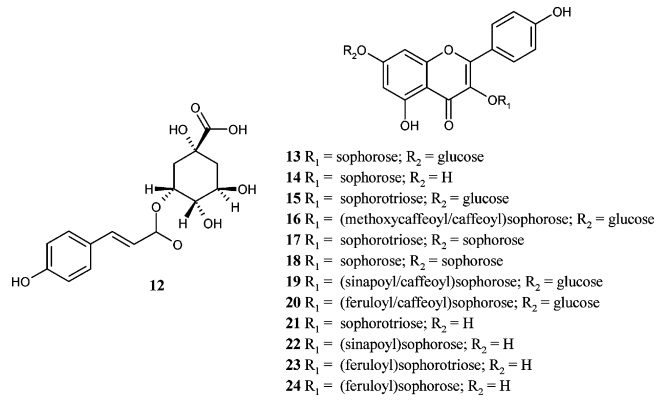

Figure 3. Chemical structures of the phenolic compounds present in tronchuda cabbage external leaves. (**12**) 3-*p*-coumaroylquinic acid; (**13**) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (**14**) kaempferol 3-*O*-sophoroside; (**15**) kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside; (**16**) kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside; (**17**) kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside; (**18**) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside; (**19**) kaempferol 3-*O*-(sinapoyl/caffeoyl)sophoroside-7-*O*-glucoside; (**20**) kaempferol 3-*O*- (feruloyl/caffeoyl)sophoroside-7-*O*-glucoside; (**21**) kaempferol 3-*O*-sophorotrioside; (**22**) kaempferol 3-*O*-(sinapoyl)sophoroside; (**23**) kaempferol 3-*O*-(feruloyl)sophorotrioside; (**24**); kaempferol 3-*O*-(feruloyl)sophoroside.

The internal leaf extract presented both flavonol glycosides and hydroxycinnamic acid esters: 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside, sinapoyl glucoside acid, kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside, 4-*p*-coumaroylquinic acid, sinapic acid, kaempferol 3-*O*-sophoroside, 1,2-disinapoylgentiobiose, 1,2,2′-trisinapoylgentiobiose and 1,2′-disinapoyl-2-feruloylgentiobiose (**Figure 4**). These compounds were also described previously in tronchuda cabbage internal leaves (*15, 17*). This extract exhibited the lowest phenolics content (ca. 1.4 g/kg), with kaempferol derivatives representing ca. 54% of total compounds (**Table 2**).

Concerning organic acids, the qualitative profile of the different plant material was identical, being composed of aconitic, citric, ascorbic, malic, quinic, shikimic, and fumaric acids (**Figure 5**). All these compounds were already reported in tronchuda cabbage (*14–19*). Seed extract consisted of ca. 16 g/kg of total identified compounds, while internal and external leaves showed a slightly higher amount (ca. 23 g/kg and 25 g/kg, respectively). Despite this, ascorbic acid was the major compound in both seed (ca. 52%) and external leaf (ca. 34%) extracts and an important one in the internal leaf extract (ca. 26%) (**Table 3**).

In order to establish a possible correlation between the chemical constitution of tronchuda cabbage extracts and the protective effects observed against NO and $ONOO^-$, sinapic acid and kaempferol 3-*O*-rutinoside were tested, since none of the identified phenolic acids or kaempferol derivatives were commercially available. Ascorbic acid was also assayed because of its predominance in the extracts. For the three tested compounds, a concentration dependent scavenging activity was observed against the two RNS (**Figure 6**), sinapic acid being the most effective one in both cases (**Table 4**). In fact, this compound exhibited a NO scavenging effect comparable to that of rutin and an activity against $ONOO^-$ stronger than that of DL-penicillamine, the two positive controls used in the assays.

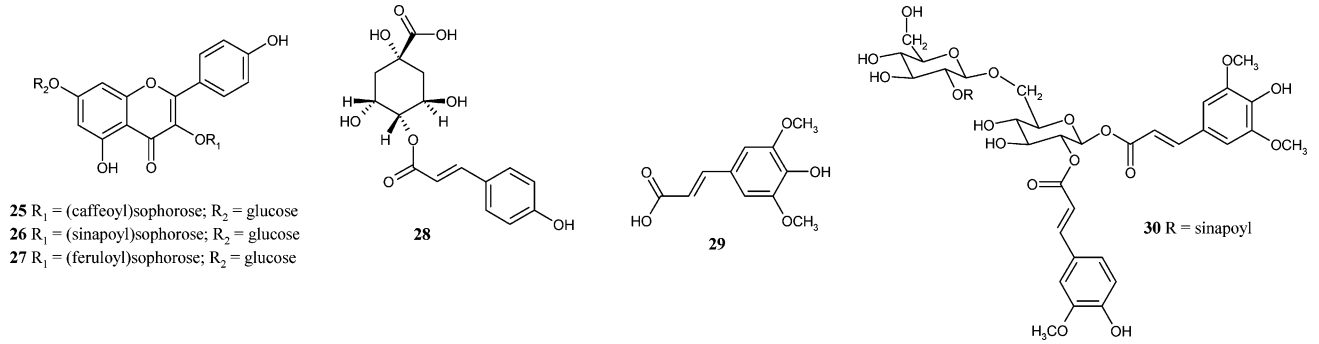

Figure 4. Chemical structures of several phenolic compounds present in tronchuda cabbage internal leaves. Kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside (**25**); kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside (**26**); kaempferol 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside (**27**); 4-*p*-coumaroylquinic acid (**28**); sinapic acid (**29**); 1,2′-disinapoyl-2-feruloylgentiobiose (**30**).

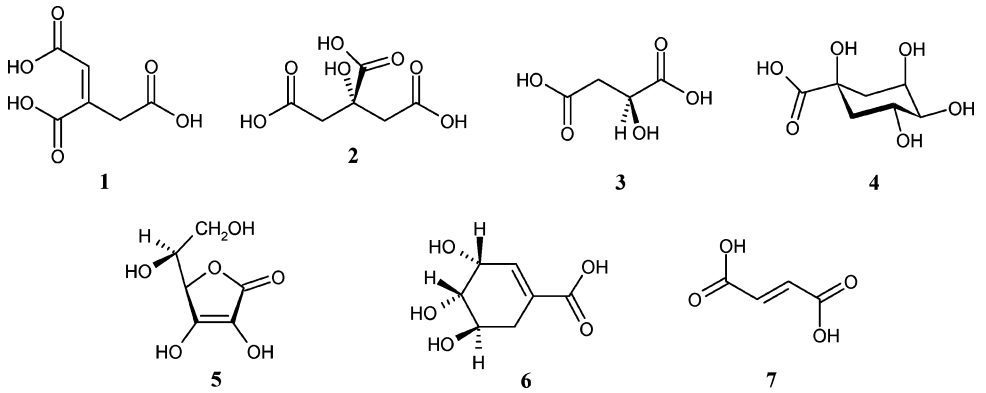

Figure 5. Chemical structures of the organic acids present in tronchuda cabbage materials. Aconitic acid (**1**); citric acid (**2**); malic acid (**3**); quinic acid (**4**); ascorbic acid (**5**); shikimic acid (**6**); fumaric acid (**7**).

Table 3. Quantification of Organic Acids (mg/kg, dry basis)[a]

	compound						
sample	aconitic	citric	malic + quinic	ascorbic	shikimic	fumaric	Σ
seeds	17 ± 2.5	4685 ± 196.9	3049 ± 221.6	8546 ± 438.4	18 ± 0.4	39 ± 0.5	16507
internal leaves	191 ± 3.7	9975 ± 68.2	6626 ± 164.8	6020 ± 143.4	35 ± 1.0	408 ± 1.8	23255
external leaves	22 ± 8.2	8131 ± 421.2	8605 ± 974.6	8754 ± 517.4	20 ± 0.5	14 ± 0.1	25545

[a] Results are expressed as mean of three determinations. SD standard deviation, Σ sum of the determined organic acids.

The strong protective effect of sinapic acid against NO and $ONOO^-$ has already been observed in other systems and an electron donation mechanism is involved (*5, 30*). These findings are in good agreement with those obtained with tronchuda cabbage seed extract once this is the extract with the highest sinapic acid derivatives content (**Table 2**).

Ascorbic acid exhibited the weakest NO scavenging ability, but a higher antioxidant potential was obtained against $ONOO^-$-induced tyrosine nitration (**Figure 6**, **Table 4**). In fact, the reactivity of ascorbic acid toward peroxynitrite through two intermediate forms is well documented in kinetic studies (*31–33*). Kaempferol 3-*O*-rutinoside showed the lowest capacity to inhibit 3-nitrotyrosine formation, but its scavenging activity against NO was higher than that from ascorbic acid (**Figure 6**, **Table 4**). These results suggest that flavonol glycosides contribute to the effects observed with tronchuda cabbage external leaves, in which this class of phenolic compounds is predominant, its content being higher than that found in the internal leaf extract (**Table 2**). As far as we know, this is the first time that a kaempferol derivative is studied for its RNS scavenging capacity.

Although the scavenging activity of phenolic compounds and organic acids was demonstrated, it is difficult to predict how a complex mixture as that of a plant extract functions against RNS, because interactions between these and other compounds present in the extract may potentiate or prevent the expected activity. Consequently, it seems important and most realistic to evaluate the activity of the extracts as a whole, as the antioxidant capacity exhibited is the resulting sum effect of the several compounds present, belonging to different chemical classes. It should be noticed that, as mentioned above, tronchuda cabbage is widely used in soups, which requires a preparation similar to that of the extracts used in the present work. So, those soups would be expected to have the same effect as that of the analyzed extracts.

In conclusion, the results obtained in the present study show that tronchuda cabbage can effectively scavenge reactive nitrogen species, and these antioxidant properties are concentration dependent. These findings, in addition to those found previously for reactive oxygen species, may explain, at least in part, the protective effect of tronchuda cabbage against free radical-mediated diseases, namely

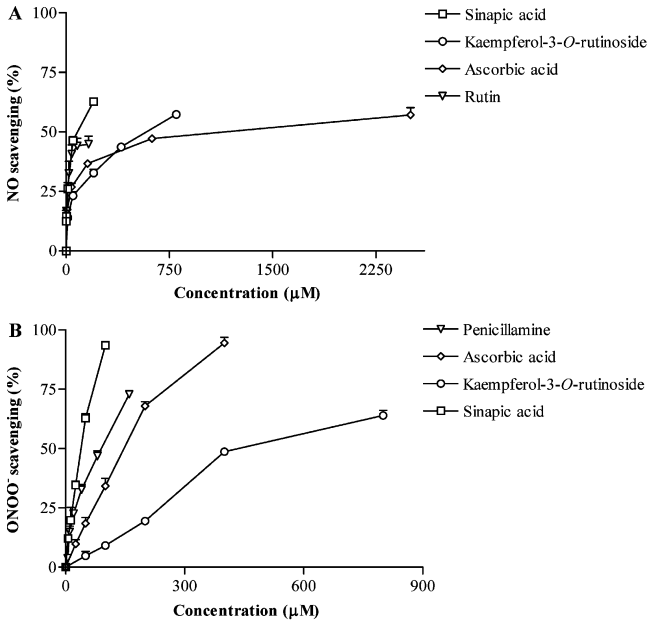

Figure 6. Effect of standards and reference compounds against (**A**) nitric oxide and (**B**) peroxynitrite. Values show mean + SE from three experiments, performed in triplicate.

Table 4. IC_{50} Values ± SE (μM) of Standard and Reference Compounds against Reactive Nitrogen Species

compound	NO	ONOO
ascorbic acid	1301 ± 412	146 ± 6
sinapic acid	79 ± 11	39 ± 1
kaempferol 3-*O*-rutinoside	567 ± 25	430 ± 20
rutin	14.0 ± 4[a]	-
DL-penicillamine	-	89 ± 7

[a] IC_{25} value.

cancer. It should also be taken into account that especially tronchuda cabbage seed extracts could be used as natural antioxidants or to functionalize foodstuffs, since this material is not eaten and displayed the strongest antioxidant potential. If an extract does not show antioxidant activity *in vitro*, then it will certainly not exhibit this capacity *in vivo*. Further studies concerning their *in vivo* activity and bioavailability are necessary before incorporating seed extracts as a dietary complement.

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4.14. Screening of antioxidant phenolic compounds roduced by i*n vitro* shoots of *Brassica oleracea* L. var. *costata* DC

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Screening of Antioxidant Phenolic Compounds Produced by *In Vitro* Shoots of *Brassica oleracea* L. var. *costata* DC

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Abstract: The phenolic compounds produced by *in vitro* shoots of *Brassica oleracea* L. var. *costata* DC were screened by LC-DAD-MS/MS. Thirty seven compounds were characterized, which included chlorogenic acids, flavonoids (the majority of them were hydroxycinnamic acid esters of kaempferol and quercetin glycosides) and hydroxycinnamic acyl glycosides (with predominance of synapoyl gentiobiosides). The antioxidant capacity of the shoots was assessed against DPPH radical and two reactive oxygen species (superoxide radical and hypochlorous acid). A strong concentration-dependent antioxidative capacity was verified in the DPPH and superoxide radicals assays, but a reduced effect was noticed against hypochlorous acid. The results obtained indicate that the *in vitro* production of *B. oleracea* var. *costata* shoots can become important in the obtention of a noticeable dietary source of compounds with health protective potential.

Keywords: *Brassica oleracea* L. var. *costata* DC, *in vitro*, shoots, phenolic compounds, antioxidant activity.

1. INTRODUCTION

Brassica oleracea L. is one of the most important human food crop plants, which have been bred into a wide range of cultivars, such as broccoli, Brussels sprouts, cauliflower and kale [1]. *Brassica oleracea* L. var. *costata* DC, commonly known as tronchuda cabbage, is a unique vegetable important for Portuguese horticulture, owing to its excellent adaptation to a wide range of climatic conditions and good integration in the traditional small farming cropping systems.

In recent years, considerable attention has been directed towards the identification of natural antioxidants, namely those plant derived, that may be used for human consumption regarding health promotion and disease prevention. Among phytochemicals possessing antioxidant capacity, phenolic compounds are one of the most important groups [2]. The polyphenol composition of several parts from field grown *B. oleracea* var. *costata* has been described, revealing distinct qualitative and quantitative profiles between them. Complex quercetin, kaempferol and phenolic acids derivatives were found in its external and internal leaves, seeds and sprouts [3-7], and the different composition seems to be determinant for the antioxidant activity displayed by each part in several systems [5, 6, 8].

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The most applicable use of tissue culture is in the area of propagation, in order to produce plants of selected quality and uniformity, usually in large quantities. The advantages include the continuous production of plants in response to market requirements, the propagation of difficult species, the propagation of plants at rates far in excess of conventional means, and the eradication of diseases since meristems are frequently free of microorganisms [9].

Growth and development of *in vitro* plant tissue depend on the composition of the nutrient medium, among other factors. Higher plant hormone regulators, especially auxins and cytokinins, are very significant in *in vitro* cultures. Auxins generally cause cell elongation and swelling of tissues, cell division and the formation of adventitious roots, and the inhibition of adventitious and axillary shoot formation. Cytokinins promote cell division and shoot growth and are used to stimulate growth and development [9, 10]. In addition, gibberellins are phytoregulators considered to be key regulators of many aspects of vegetative and reproductive development, including stem elongation, leaf differentiation and photomorphogenesis [11]. Generally, gibberellins induce elongation of internodes and the growth of meristems or buds *in vitro*, and usually inhibit adventitious root and adventitious shoot formation [10].

The accumulation of phenolics in *in vitro* cultures is known to be influenced by supplementation with growth regulators. As a consequence, the phenolic composition can be modulated in order to achieve the best antioxidative properties [12-14]. Several studies have demonstrated that the

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development of shoots from *B. oleracea* occurs in media containing both cytokinins and auxins [1, 15-17], but none of them involved the *costata* variety. As far as we know, there have been no studies on the production of pharmacologically interesting phenolic compounds by *in vitro* cultures of *B. oleracea* var. *costata*. Thus, the objective of this work was to characterize the phenolic compounds produced by shoots of *B. oleracea* var. *costata* and the evaluation of the antioxidant potential of this material. For this purpose, phenolics were screened by LC-DAD-MS/MS, and the antioxidant capacity was assessed against DPPH and superoxide ***radicals and hypochlorous acid.***

2. MATERIALS AND METHODS

2.1. Reagents

Acetic and hydrochloric acids, sodium hydroxide and methanol were obtained from Merck (Darmstadt, Germany). Phenazine methosulfate (PMS), β-nicotinamide adenine dinucleotide (NADH), DPPH, nitroblue tetrazolium chloride (NBT), sodium hypochlorite solution with 4% available chlorine (NaOCl), and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. (St. Louis, USA). The water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Plant Material and Sterilization

Brassica oleracea var. *costata* seeds were dipped in a 90% ethanol solution for 10 min, followed by washing with sterilized water, before surface sterilization with a 5% sodium hypochlorite solution for 10 min. After rinsing with sterilized water, seeds were germinated on autoclaved MS [18] basal medium, devoid of growth regulators, with 20 g/L sucrose and solidified with 8 g/L agar. Seeds germination was performed in a growth chamber in complete darkness for five days and then under 16 h light and 23 ºC / 8h dark and 16 ºC cycle.

2.3. *In vitro* Shoots Culture

Internodal shoot segments (~ 10 mm), obtained from aseptic 3 weeks seedlings, were used as primary explants in the development of shoot cultures on MS basal medium supplemented with 20 g/L sucrose, benzylaminopurine (BAP) 2 mg/L, naphthaleneacetic acid (NAA) 0.1 mg/L, and gibberellic acid (GA) 0.5 µM. The culture conditions were those described above, and shoots were subcultured in the same medium at intervals of 3 weeks. At the end of the third subculture period, shoots were withdrawn and lyophilized for seven days.

2.4. Phenolic Compounds Extraction

For the identification of the phenolic compounds in shoots, the dried powdered material (ca. 0.1 g) was thoroughly mixed with 1 mL water, ultra-sonicated (1 h), macerated for 18 h, and ultra-sonicated again (1 h). The resulting extract was then centrifuged (12000 rpm, 5 min), and the supernatant was collected and filtrated trough a 0.45 µm size pore membrane. For the screening of antioxidant activity, 0.43 g of dried material were boiled for 15 min in 300 mL of water and then filtered using a Büchner funnel. The resulting extract was lyophilized, and a yield of 0.17 g was obtained. The lyophilized extract was kept in an dessicator in the dark.

2.5. Alkaline Hydrolysis

For the study of the acyl flavonoids, alkaline hydrolysis was performed followed by mass spectrometric analysis of the deacylated derivatives. This hydrolysis procedure was necessary since losses of 146 u for *p*-coumaroyl moieties and of 162 for caffeoyl residues coincide with those of rhamnosyl and hexosyl residues, respectively. Otherwise, a misassignment of the mass spectrometric data might occur.

Saponification was performed by alkalinizing the extract (1 mL) with 2 M NaOH (up to pH 9–10) and keeping the mixture for 12 h at room temperature in a stopper test tube under N_2 atmosphere. After this step, the alkaline hydrolysis products were acidified with concentrated HCl (up to pH 1–2) and directly analysed by HPLC-UV diode array detection (DAD)-electrospray ionization mass spectrometry.

2.6. HPLC-DAD-MS/MS Analysis

Chromatographic separations were carried out on a 250 x 4 mm, 5 µm, RP-18 LiChroCART column (Merck, Darmstadt, Germany) protected with a 4 x 4 mm LiChroCART guard column, with 1% acetic acid (A) and methanol (B) as solvents, starting with 20% B and using a gradient to obtain 50% B at 30 min, and 80% B at 37 min. The flow rate was 1 mL/min, and the injection volume was 20 µL. The HPLC system was equipped with an Agilent 1100 Series diode array absorbance detector, and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser and a G1315B photo-diode array detector, controlled by ChemStation software (Agilent, v. 08.03). Spectroscopic data from all peaks were accumulated over the range 240-400 nm, and chromatograms were recorded at 320 nm.

The mass detector was a G2445A ion-trap mass spectrometer equipped with an electrospray ionisation system and controlled by LCMSD software (Agilent, v. 4.1.). Nitrogen was used as the nebulising gas at a pressure of 65 psi, and the flow was adjusted to 11 L/min. The heated capillary and voltage were maintained at 350 ºC and 4 kV, respectively. Mass spectra were recorded over the range *m/z* 100-2000. Collision-induced dissociationwas performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS data were acquired using the negative ionization mode. The classical nomenclature for glycoconjugates [19] was adopted to designate the ions obtained from the fragmentation of flavonoid glycosides (Table **2**). The ions $\mathbf{Y^n_j}$ and $\mathbf{Z^n_j}$ ($\mathbf{Y^n_j\text{-}H_2O}$) represent those fragments still containing the flavonoid aglycone, where **j** is the number of the interglycosidic bonds broken, counted from the aglycone; and **n** represents the position where the oligosaccharide is attached to the aglycone. In this context, the product ions obtained by MS^3 have been labelled starting with the precursor ion and followed by the product ion, *e.g.*, the ion $[Y^7_0Y^3_2]^-$ denotes the ion formed from the fragmentation of ion $Y^7_0{}^-$ (loss of a glycosylation in the 7 position, MS^3 ($[M\text{-}H]^- \rightarrow Y^7_0{}^-$) by loss of the terminal glucose (-162) of the triglucoside in the 3 position. The losses indicated in the MS^3

Table 1. *R*t, UV and MS: $[M-H]^-$, $-MS^2$ $[M-H]^-$ Data of Monoacyl Chlorogenic Acids[a]

Compounds[b]		*R*t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$-MS^2$ $[M-H]^-$ (*m/z*) (%)		
					$[Quinic\ acid-H]^-$	$[Cinnamoyl-H]^-$	$[191-18]^-$
1	3-CQA	4.2	325, 300sh	353	191(100)	179(39)	
2	3-*p*-CoQA	5.7	311	337	191(6)	163(100)	
3	3-FQA[c]	6.5	---	367		193(100)	
4	4-CQA[c]	6.8	---	353	191(42)	179(70)	173(100)
11	4-*p*-CoQA[c]	9.5	---	337			173(100)

[a] Main observed fragments. Other ions were found but they have not been included.
[b] CQA: caffeoylquinic acid; *p*-CoQA: *p*-coumaroylquinic acid; FQA: feruloylquinic acid.
[c] Compounds hidden by others. Their UV spectra were properly observed.

spectrum of the fragment came from the trapped and fragmented intermediate ion and not from the deprotonated molecule. This terminology complements the nomenclature provided by Domon and Costello [19] by addition of specific ways the ions are obtained using MS^3.

Tables **1-5** show the most frequent ions which characterize the fragmentation of the compounds. Other ions were found, but they have not been included due to the lack of space on the tables and their low significance on the MS behaviour ions. The UV spectra of some compounds have not been observed properly because they were hidden by others or present in traces. Tables **2-4** have been designed taking into account the type of glycosylation or the nature of the aglycone. Table **5** (acyl gentiobiosides) was divided in three groups, starting with those derivatives presenting the highest number of sinapoyl residues within each one. These groups have been ordered according to an increasing retention time (*R*t).

2.7. DPPH• Scavenging Activity

The antiradical activity of the extract was determined spectrophotometrically in a Multiskan Ascent plate reader (Thermo Electron) by monitoring the disappearance of DPPH• at 515 nm, according to a described procedure [5, 6, 8]. The reaction mixture in the sample wells consisted of 25 µL aqueous extract and 200 µL of methanolic solution of DPPH• 150 mM. The plate was incubated for 30 min at room temperature after addition of DPPH•. Three experiments were performed in triplicate.

Table 2. *R*t, UV, and MS: $[M-H]^-$, $-MS^2$ $[M-H]^-$ and $-MS^3$ $[(M-H)\rightarrow Y^7_0]^-$ Data of Glycosyl Flavonoids After Deacylation[a]

Compounds[b]		*R*t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	Flavonol-3-*O*-Glycoside-7-*O*-Glycoside					
					MS^2 $[M-H]^-$ (*m/z*)	MS^3 $[(M-H)\rightarrow Y^7_0]^-$ (*m/z*) (%)				
					$[Y^7_0]^-$	$[Y^7_0Y^3_2]^-$	$[Y^7_0Z^3_2]^-$	$[Y^7_0Y^3_1]^-$	$[Y^7_0Z^3_1]^-$	$[Aglc-2H/H]^-$
A	Q-3-tG-7-G[c]	5.6	---	949	787	625(90)			445(30)	300(100)
B	Q-3-tG-7-dG[c]	5.8	---	1111	787	625(22)			445(76)	300(100)
C	Q-3-dG-7-G	6.3	255, 267sh, 349	787	625				445(25)	300(100)
D	K-3-tG-7-G	6.9	267, 317sh, 348	933	771	609(27)	591(5)		429(11)	285(100)
E	K-3-tG-7-dG[c]	7.4	---	1095	771	609(100)			429(60)	285(90)
F	K-3-dG-7-G[c]	7.4	---	771	609			447(9)	429(40)	285(100)
G	K-3-tG-7-R	13.9	267, 313sh, 350	917	771	609(25)			429(11)	285(100)
					Flavonol-3-*O*-glycoside					
					MS^2 $[M-H]^-$ (*m/z*) (%)					
						$[Y^3_2]^-$	$[Z^3_2]^-$	$[Y^3_1]^-$	$[Z^3_1]^-$	$[Aglc-2H/H]^-$
H	Q-3-tG[c]	15.1	---	787		625(84)		463(41)	445(100)	300(85)
18	Q-3-dG[c]	16.1	---	625				463(17)	445(28)	300(100)
J	K-3-tG	18.1	265, 301sh, 349	771		609(59)	591(50)	447(11)	429(64)	285(100)
23	K-3-dG	19.5	266, 301sh, 349	609				447(7)	429(50)	285(100)

[a] Main observed fragments. Other ions were found but they have not been included.
[b] Q: quercetin; K: kaempferol; G: glucose; R: rhamnose; Q-3-tG-7-G: quercetin-3-*O*-triglucoside-7-*O*-glucoside.
[c] Compounds hidden by others or in traces. Their UV spectra were not properly observed.

Table 3. ***R*t, MS: [M-H]$^-$, -MS^2 [M-H]$^-$ and -MS^3 [(M-H)→(M-H-Glyc7)]$^-$ Data of Flavonol-3-*O*-(Monoacyl)-Triglucoside-7-*O*-Glycosides and Flavonol-3-*O*-(Monoacyl)-Triglucosides[a]**

Compounds[b, c]		*R*t (min)	[M-H]$^-$ (*m/z*)	MS^2 [M-H]$^-$ (*m/z*) (%)				MS^3 [(M-H)→(M-H-Gly7)]$^-$ (*m/z*) (%)		
				Flavonol-3-*O*-(monoacyl)-triglucoside-7-*O*-glucoside						
				-Glc (-162)	**-Glc-Caf (-162-162)**	**-Glc-Fer (-162-176)**	**-Glc-Sinp (-162-206)**	**-Caf (-162)**	**-Fer (-176)**	**-Sinp (-206)**
7	Q-3-S-tG-7-G	7.9	1155	993(90)			787(100)			787(100)
5	K-3-C-tG-7-G	7.3	1095	933(100)	771(22)			771(100)		
10	K-3-S-tG-7-G	9.1	1139	977(100)			771(2)			771(100)
12	K-3-F-tG-7-G	9.8	1109	947(100)		771(6)			771(100)	
				Kaempferol-3-*O*-(monoacyl)-triglucoside-7-*O*-rhamnoside						
				-Rhmn (-146)		**-Rhmn-Fer (-146-176)**	**-Rhmn-Sinp (-146-206)**		**-Fer (-176)**	**-Sinp (-206)**
14	K-3-S-tG-7-R	13.9	1123	977(100)			771(20)			771(100)
17	K-3-F-tG-7-R	15.1	1093	947(100)		771(11)			771(100)	
				Kaempferol-3-*O*-(monoacyl)-triglucoside-7-*O*-diglucoside						
				-diGlc (-324)			**-diG-Sinp (-530)**			**-Sinp (-206)**
9	K-3-S-tG-7-dG	8.9	1301	977(100)			771(55)			771(100)
				Flavonol-3-*O*-(monoacyl)-triglucoside						
				-Caf (-162)	**-Fer (-176)**	**-MeOCaf (-192)**	**-Sinp (-206)**			
15	Q-3-S-tG	14.2	993				787(100)			
16	K-3-MC-tG	14.7	963			771(100)				
20	K-3-C-tG	16.3	933	771(100)						
21	K-3-S-tG	16.9	977				771(100)			
22	K-3-F-tG	18.5	947		771(100)					

[a] Principal fragments observed. Other ions were found but they have not been included.
[b] Q: quercetin. K: kaempferol. G: Glc: glucoside, dG: diGlc: diglucoside, tG: triglucoside. R: Rhmn: rhamnoside. C: Caf: caffeoyl. MC: MeOCaf: methoxycaffeoyl. F: Fer: Feruloyl. S: Sinp: sinapoyl.
[c] The UV spectra were not properly observed since they were co-eluting with other compounds or present in trace amounts, with the exceptions of compounds (**5**) 269, 325 nm and (**16**) 270sh, 325 nm.

2.8. Evaluation of Superoxide Radical Scavenging Activity

Antiradical activity was determined spectrophotometrically by monitoring the effect of the lyophilized extract on the superoxide radical-induced reduction of NBT at 562 nm. Superoxide radicals were generated by the NADH/PMS system according to a described procedure [5, 6, 8]. All components were dissolved in phosphate buffer (19 mM, pH 7.4). Three experiments were performed in triplicate.

2.8. Hypochlorous Acid Scavenging Activity

The inhibition of hypochlorous acid-induced 5-thio-2-nitrobenzoic acid (TNB) oxidation to 5,5'-dithiobis(2-nitrobenzoic acid) was performed according to a described procedure [5, 6, 8], in a double beam spectrophotometer (Heλios α, Unicam). Hypochlorous acid and TNB were prepared immediately before use. Three experiments were performed in triplicate.

3. RESULTS AND DISCUSSION

Shoots of *Brassica oleracea* var. *costata* were induced from shoot internode explants on MS medium under the effect of hormonal supplementation with an auxin (NAA), a cytokinin (BAP) and gibberellic acid which have been used in the *in vitro* propagation of other *B. oleracea* parts [1, 15-17].

3.1. Characterization of Phenolic Compounds from Shoots

The LC-DAD-MS/MS screening of the crude aqueous extract of *Brassica oleracea* var. *costata* shoots showed the presence of a high number of phenolic compounds (Fig. **1A**).

Table 4. ***R*t, MS: [M-H]$^-$, MS^2 [M-H]$^-$, MS^3 [(M-H)→(M-H-Glyc7)]$^-$ and MS^3 [(M-H)→(M-H-Glyc7-Acyl)]$^-$ Data of Flavonol-3-*O*-(diacyl)-Triglucoside-7-*O*-Glycosides[a]**

Compounds[b, c]		*R*t (min)	[M-H]$^-$ (*m/z*)	MS^2 [M-H]$^-$ (*m/z*) (%)				MS^3 [(M-H)→(M-H-Gly7)]$^-$ (*m/z*) (%)						MS^3 [→(-Gly7-Acyl)]$^-$ (*m/z*) (%)
				Flavonol-3-*O*-(Diacyl)-Triglucoside-7-*O*-Glucoside										
				-Glc (-162)	-G-C (-324)	-G-MC (-354)	-G-S (-368)	-C (-162)	-MC (-192)	-S (-206)	-C-S (-368)	-MC-S (-398)	-S-S (-412)	
29	Q-3-dS-tG-7-G	21.8	1361	1199 (95)			993 (100)			993 (100)			787 (28)	787 (100)
26	K-3-MC/ S-tG-7-G	20.5	1331	1169 (100)		977 (25)	963 (3)		977 (100)			771 (23)		771 (100)
28	K-3-C/ S-tG-7-G	21.5	1301	1139 (100)	977 (10)		933 (2)	977 (100)		933 (7)	771 (50)			771 (100)
32	K-3-dS-tG-7-G	22.9	1345	1183 (100)			977 (13)			977 (100)			771 (51)	771 (100)
				Kaempferol-3-*O*-(diacyl)-triglucoside-7-*O*-diglucoside										MS^3 [→(-diGly-Acyl)]$^-$ (*m/z*) (%)
				MS^2 [M-H]$^-$ (*m/z*) (%)				MS^3 [(M-H)→(M-H-diGlc)]$^-$ (*m/z*) (%)						
				-diGlc (-324)	-dG-C (-486)	-dG-MC (-516)	-diG-S (-530)	-C (-162)	-MC (-192)	-S (-206)	-C-S (-368)	-MC-S (-398)		
24	K-3-MC/ S-tG-7-dG	19.9	1493	1169 (100)		977 (51)	963 (10)		977 (82)	963 (100)		771 (98)		771 (100)
27	K-3-C/ S-tG-7-dG	21.1	1463	1139 (100)	977 (40)		933 (8)	977 (100)			771 (70)			771 (100)

[a] Principal fragments observed. Other ions were found but they have not been included.
[b] Q: quercetin;. K: kaempferol; G: Glc: glucoside; dG: diGlc: diglucoside; tG: triglucoside; C: caffeoyl; MC: methoxycaffeoyl; S: Sinp: sinapoyl.
[c] The UV spectra were not properly observed since they were co-eluting with other compounds or present in trace amounts, with the exceptions of compounds (**26**) 269, 329 nm and (**27**) 270, 329 nm.

Table 5. ***R*t; UV, and MS: [M-H]$^-$, MS^2 [M-H]$^-$ and MS^3 [(M-H)→(M-H-224)]$^-$ Data of Hydroxycinnamic Acyl Gentiobiosides[a]**

Compounds[b]		*R*t (min)	UV (nm)	[M-H]$^-$ (*m/z*)	MS^2 [M-H]$^-$ (*m/z*) (%)			MS^3 [(M-H)→(M-H-224)]$^-$ (*m/z*) (%)			
					diAcyl-Gentiobioside						
					-194	-224	[Sinp-H]$^-$		[Sinp-H]$^-$	[Fer-H]$^-$	[Acyl-H-18]$^-$
13	diSinp-Gentb	10.8	327	753		529(100)	223(4)		223(100)		205(58)
33	diSinp-Gentb	24.1	329	753		529(100)	223(8)		223(60)		205(100)
25	SinpMeOCaff-Gentb	20.4	---	739		515(100)					191(100)
34	SinpFer-Gentb	25.0	329, 301sh	723	529(9)	499(100)				193(100)	175(21)
					triAcyl-Gentiobioside						
						-224	-224-206	-192	-206	-224	[Sinp-H]$^-$
35	triSinp-Gentb[c]	25.8	326	959		735(100)	529(7)		529(46)	511(100)	223(27)
30	diSinpMeOCaff-Gentb	22.2	333	945		721(100)	515(14)	529(22)	515(100)	497(64)	223(6)
31	diSinpCaff-Gentb	22.9	---	915		691(100)	485(12)		485(100)	467(4)	223(10)
36	diSinpFer-Gentb	26.5	327	929		705(100)	499(5)		499(100)	481(42)	
					Other triAcyl-Gentiobioside						
					MS^2 [M-H]$^-$ (*m/z*) (%)			MS^3 [(M-H)→(base peak)]$^-$			
19	SinpCaffFer-Gentb[c]	15.9	---	885	723(100) (-162)		499(24) (-162-224)			499(100) (-224)	223(25)
37	SinpdiFer-Gentb	27.6	327, 299sh	899	705(100) (-194)	675(22) (-224)		529(35) (-176)	499(100) (-206)		223(45)

[a] Main observed fragments. Other ions were found but they have not been included.
[b] Gentb: gentiobiosyl; Sinp: sinapoyl; MeOCaff: methoxycaffeoyl; Caff: caffeoyl; Fer: feruloyl.
[c] Compounds hidden by others or in trace amounts. Their UV spectra were not properly observed.

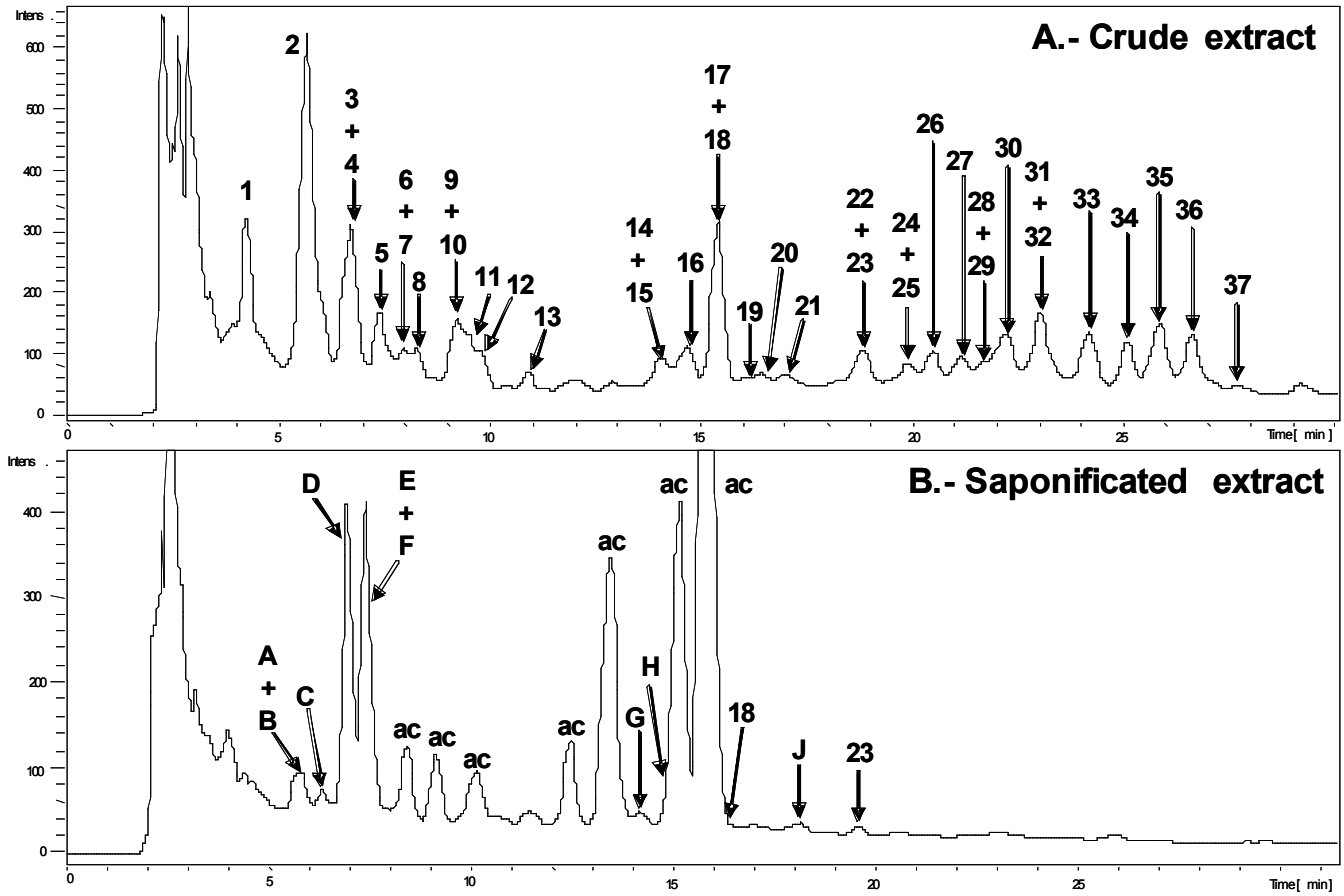

Fig. (1). HPLC phenolic profile of *B. oleracea* var. *costata* shoots. Detection at 320 nm. Peaks: (**1**) 3-caffeoylquinic acid; (**2**) 3-*p*-coumaroylquinic acid; (**3**) 3-feruloylquinic acid; (**4**) 4-caffeoylquinic acid; (**5**) kaempferol-3-*O*-(caffeoyl)-sophorotrioside-7-*O*-glucoside; (**6**) caffeoylferuloylquinic acid; (**7**) quercetin-3-*O*-(sinapoyl)-sophorotrioside-7-*O*-glucoside; (**8**) sinapoyl glucoside; (**9**) kaempferol-3-*O*-(sinapoyl)-sophorotrioside-7-*O*-sophoroside; (**10**) kaempferol-3-*O*-(sinapoyl)-sophorotrioside-7-*O*-glucoside; (**11**) 4-*p*-coumaroylquinic acid; (**12**) kaempferol-3-*O*-(feruloyl)-sophorotrioside-7-*O*-glucoside; (**13**) disinapoyl-gentiobioside; (**14**) kaempferol-3-*O*-(sinapoyl)-sophorotrioside-7-*O*-rhamnoside; (**15**) quercetin-3-*O*-(sinapoyl)-sophorotrioside; (**16**) kaempferol-3-*O*-(methoxycaffeoyl)-sophorotrioside; (**17**) kaempferol-3-*O*-(feruloyl)-sophorotrioside-7-*O*-rhamnoside; (**18**) quercetin-3-*O*-sophoroside; (**19**) sinapoylcaffeoylferuloyl-gentiobioside; (**20**) kaempferol-3-*O*-(caffeoyl)-sophorotrioside; (**21**) kaempferol-3-*O*-(sinapoyl)-sophorotrioside; (**22**) kaempferol-3-*O*-(feruloyl)-sophorotrioside; (**23**) kaempferol-3-*O*-sophoroside; (**24**) kaempferol-3-*O*-(methoxycaffeoyl/sinapoyl)-sophorotrioside-7-*O*-sophoroside; (**25**) sinapoylmethoxycaffeoyl-gentiobioside; (**26**) kaempferol-3-*O*-(methoxycaffeoyl/sinapoyl)-sophorotrioside-7-*O*-glucoside; (**27**) kaempferol-3-*O*-(caffeoyl/sinapoyl)-sophorotrioside-7-*O*-sophoroside; (**28**) kaempferol-3-*O*-(caffeoyl/sinapoyl)-sophorotrioside-7-*O*-glucoside; (**29**) quercetin-3-*O*-(disinapoyl)-sophorotrioside-7-*O*-glucoside; (**30**) disinapoylmethoxycaffeoyl-gentiobioside; (**31**) disinapoyl-caffeoyl-gentiobioside; (**32**) kaempferol-3-*O*-(disinapoyl)-sophorotrioside-7-*O*-glucoside; (**33**) disinapoyl-gentiobioside isomer; (**34**) sinapoylferuloyl-gentiobioside; (**35**) trisinapoyl-gentiobioside; (**36**) disinapoylferuloyl-gentiobioside; (**37**) sinapoyldiferuloyl-gentiobioside; (**A**) quercetin-3-*O*-sophorotrioside-7-*O*-glucoside; (**B**) quercetin-3-*O*-sophorotrioside-7-*O*-sophoroside; (**C**) quercetin-3-*O*-sophoroside-7-*O*-glucoside; (**D**) kaempferol-3-*O*-sophorotrioside-7-*O*-glucoside; (**E**) kaempferol-3-*O*-sophorotrioside-7-*O*-sophoroside; (**F**) kaempferol-3-*O*-sophoroside-7-*O*-glucoside; (**G**) kaempferol-3-*O*-sophorotrioside7-*O*-rhamnoside; (**H**) quercetin-3-*O*-sophorotrioside; (**J**) kaempferol-3-*O*-sophorotrioside; (**ac**) compounds with hydroxycinnamic acid UV spectra–like.

Their UV spectra indicated the presence of hydroxycinnamic acid derivatives, although many of them co-eluted, and the UV information is scarce. From the MS/MS study, several groups of compounds were detected including chlorogenic acids, flavonoids (prevailing hydroxycinnamic acid esters of kaempferol and quercetin glycosides) and hydroxycinnamic acyl glycosides (with predominance of sinapoyl gentiobiosides).

Chlorogenic Acids

The deprotonated molecule $[M-H]^-$ of compounds **1**-**4** and **11** and their fragmentation patterns (Table **1**) point to monochlorogenic acid derivatives. The fragmentation of compound **1** produced a base peak at *m/z* 191 [quinic acid-H]$^-$ and an abundant ion [caffeoyl-H]$^-$ (*m/z* 179, 39%), which is accordance with 3-caffeoylquinic acid [20]. The ions [cinnamoyl-H]$^-$ at *m/z* 163 and 193 as base peaks of compounds **2** and **3** confirm their structures as 3-*p*-coumaroylquinic acid and 3-feruloylquinic acid, respectively. The fragment ion of *m/z* 173 (dehydrated, deprotonated quinic acid was the base peak in the mass spectra of compounds **4** and **11**, which characterizes 4-acylchlorogenic acid derivatives and distinguishes them from the ones substituted in other positions [20].

Another chlorogenic acid derivative, caffeoylferuloylquinic acid (**6**), was observed at *R*t 7.7 min, presenting

the $[M-H]^-$ ion of *m/z* 529. Its MS^2 fragmentation showed an ion at *m/z* 367 $[FQA-H]^-$ (60%) and a base peak at *m/z* 191 $[Q-H]^-$. These ions are characteristic of these kind of compounds [20], but their relative abundances are not coincident with that of caffeoylferuloylquinic acid derivatives studied previously (3,4-, 4,5-, 3-feruloyl-5-caffeoyl-, or 3- caffeoyl - 5- feruloyl-) [20].

Flavonoids

As already mentioned, the majority of the determined flavonoids are hydroxycinnamic acid derivatives of kaempferol and quercetin glycoside. For their structure elucidation, a saponification followed by the analysis of the deacylated glycosides was performed prior to the study of the crude aqueous extract.

Deacylated glycosides. The LC-DAD-MS/MS (Fig. **1B**, Table **2**) study revealed the existence of several flavonoids that were not observed in the crude extract (compounds **A-J**) or present in were present in trace amounts (compounds **18** and **23**). From their UV spectra and MS fragmentation, it was possible to distinguish two groups of compounds: flavonol-3-*O*-glycoside-7-*O*-glycosides (**A-G**) and flavonol-3-*O*-glycosides (**H**, **18**, **J** and **23**).

In the MS^2 fragmentation of the first group of compounds (**A-G**), it was noticed that the base peak corresponded to $[Y^7{}_0]^-$ and was formed by the loss of 162 amu, hexosyl group (compounds **A**, **C**, **D** and **F**), or of 324 amu, dihexosyl group (compounds **B** and **E**) or of 146 amu, a rhamnosyl group (compound **G**), which corresponded to the glycosidic fraction in position 7 of the aglycone. In the MS^3 mass spectrum of $[(M-H)\rightarrow Y^7{}_0]^-$, fragmentation was observed at the oligosacharide linked to the hydroxyl at position 3 of the aglycone (Table **2**). The abundance of these ions indicates that the interglycosidic bond is not (1→6) but probably (1→2) instead [21]. In most cases, the deprotonated aglycone is the base peak $[Aglc-2H/H]^-$, at *m/z* 300 for quercetin and at *m/z* 285 for kaempferol.

The MS^2 fragmentation of the flavonol-3-*O*-glycosides is similar to the MS^3 of flavonol-3-*O*-glycosides-7-*O*-glycosides. Thus, with the exception of kaempferol-3-*O*-triglucoside-7-*O*-rhamnoside (**G**), the structures of these compounds are from the same type as those described in cauliflower [22], broccoli [23] and tronchuda cabbage [3, 6]. Compound **G** has been recently reported in watercress (*Nasturtium officinale* R. Br.), another Brassicaceae [24].

With respect to their chromatographic behaviour and in agreement with data of the above mentioned Brassicaceae, the increase in glycosylation not always corresponds to a lower *R*t in reversed-phase HPLC. So, the introduction of a second hexose residue on the hexose at position 7 leads to an increment of the *R*t (compounds **A**/**B**, and **D**/**E**) [22]. According to what was found in other Brassicaceae, the following compounds were characterized: quercetin-3-*O*-sophorotrioside-7-*O*-glucoside (**A**), quercetin-3-*O*-sophorotrioside-7-*O*-sophoroside (**B**), quercetin-3-*O*-sophoroside-7-*O*-glucoside (**C**), kaempferol-3-*O*-sophorotrioside-7-*O*-glucoside (**D**), kaempferol-3-*O*-sophorotrioside-7-*O*-sophoroside (**E**), kaempferol-3-*O*-sophoroside-7-*O*-glucoside (**F**), kaempferol-3-*O*-sophorotrioside7-*O*-rhamnoside (**G**), quercetin-3-*O*-sophorotrioside (**H**), quercetin-3-*O*-sophoroside (**18**), kaempferol-3-*O*-sophorotrioside (**J**) and kaempferol-3-*O*-sophoroside (**23**). The kaempferol derivatives are predominant.

Flavonoids of the crude extract. Besides the deacylated glycosides **18** and **23** described above, 12 monoacylated flavonoid glycoside derivatives (**5**, **7**, **9**, **10**, **12**, **14-17**, **20-22**) and 6 diacylated derivatives (**24**, **26-29**, **32**) were detected in the crude aqueous extract (Fig. **1A**, Tables **3** and **4**).

In the MS^2 fragmentation of the mono- and diacylated compounds deriving from flavonol-3-*O*-triglucoside-7-*O*-glycosides (-7-*O*-glucoside: **5**, **7**, **10**, **12**, **26**, **28**, **29**, **32**; -7-*O*-rhamnoside: **14**, **17**; -7-*O*-diglucoside: **9**, **24**, **27**), loss of the glycosidic fraction at position 7 was observed corresponding to -162 (glucosyl), -146 (rhamnosyl) or -324 amu (diglucosyl) (Tables **3** and **4**, Fig. **2**). This fragmentation pathway was also observed above for the deacylated compounds and formed the base peak in most cases. In this fragmentation, the monoacylated compounds could also display the simultaneous loss of that glycosidic moiety plus the acyl radical to form the ion of the aglycone glycosylated at position 3, which was observed at *m/z* 771 and 787 in the case of kaempferol and quercetin derivatives, respectively (Table **3**). This ion, corresponded to the base peak in the MS^3 spectrum $[(M-H)\rightarrow(M-H-Gly7)]^-$.

In the same way, the MS^2 fragmentation of diacylated compounds showed the simultaneous loss of the glycosidic moiety plus some of the acyl group (Table **4**, Fig. **2**). The MS^3 $[(M-H)\rightarrow(M-H-Gly7)]^-$ spectra exhibited the ions obtained from the individual losses of the remaining acyl radicals, as well as that resulting from the simultaneous loss of both acyl groups, which as described above for monoacylated compounds, corresponded to *m/z* 771 and 787 for kaempferol and quercetin derivatives, respectively (Fig. **2**). These ions will also represent the base peak in the MS^3 $[(M-H)\rightarrow(M-H-Gly7-Acyl)]^-$ spectra (Table **4**). The fragmentation of the ions at *m/z* 771 and 787 (not included in Tables **3** and **4**) is similar to that indicated in Table **2**. These compounds were characterized as kaempferol-3-*O*-(caffeoyl)-sophorotrioside-7-*O*-glucoside (**5**), quercetin-3-*O*-(sinapoyl)-sophorotrioside-7-*O*-glucoside (**7**), kaempferol-3-*O*-(sinapoyl) -sophorotrioside-7-*O*-sophoroside (**9**), kaempferol-3-*O*-(sinapoyl)-sophorotrioside-7-*O*-glucoside (**10**), kaempferol-3-*O*-(feruloyl)-sophorotrioside-7-*O*-glucoside (**12**), kaempferol-3-*O*-(sinapoyl)-sophorotrioside-7-*O*-rhamnoside (**14**), kaempferol-3-*O*-(feruloyl)-sophorotrioside-7-*O*-rhamnoside (**17**), kaempferol-3-*O*-(methoxicaffeoyl/sinapoyl)-sophorotrioside-7-*O*-sophoroside (**24**), kaempferol-3-*O*-(methoxicaffeoyl/sinapoyl)-sophorotrioside-7-*O*-glucoside (**26**), kaempferol-3-*O*-(caffeoyl/sinapoyl)-sophorotrioside-7-*O*-sophoroside (**27**), kaempferol-3-*O*-(caffeoyl/sinapoyl)-sophorotrioside-7-*O*-glucoside (**28**), quercetin-3-*O*-(disinapoyl)-sophorotrioside-7-*O*-glucoside (**29**) and kaempferol-3-*O*-(disinapoyl)-sophorotrioside-7-*O*-glucoside (**32**).

Compounds **15**, **16**, **20-22** are flavonol-3-*O*-(monoacyl)-sophorotrioside derivatives. Their MS^2 fragmentation (Table **3**) is similar to the MS^3 $[(M-H)\rightarrow(M-H-Gly7)]^-$ spectra of flavonol-3-*O*-(monoacyl)-sophorotrioside-7-*O*-glycoside derivatives and resembles the event produced after the loss of glycosylation in position 7, in which the loss of the acyl radical formed the ion of *m/z* 771 (kaempferol derivatives: **16**, **20-22**) and *m/z* 787 (quercetin derivative: **15**). The following compounds were tentatively identified: quercetin-3-

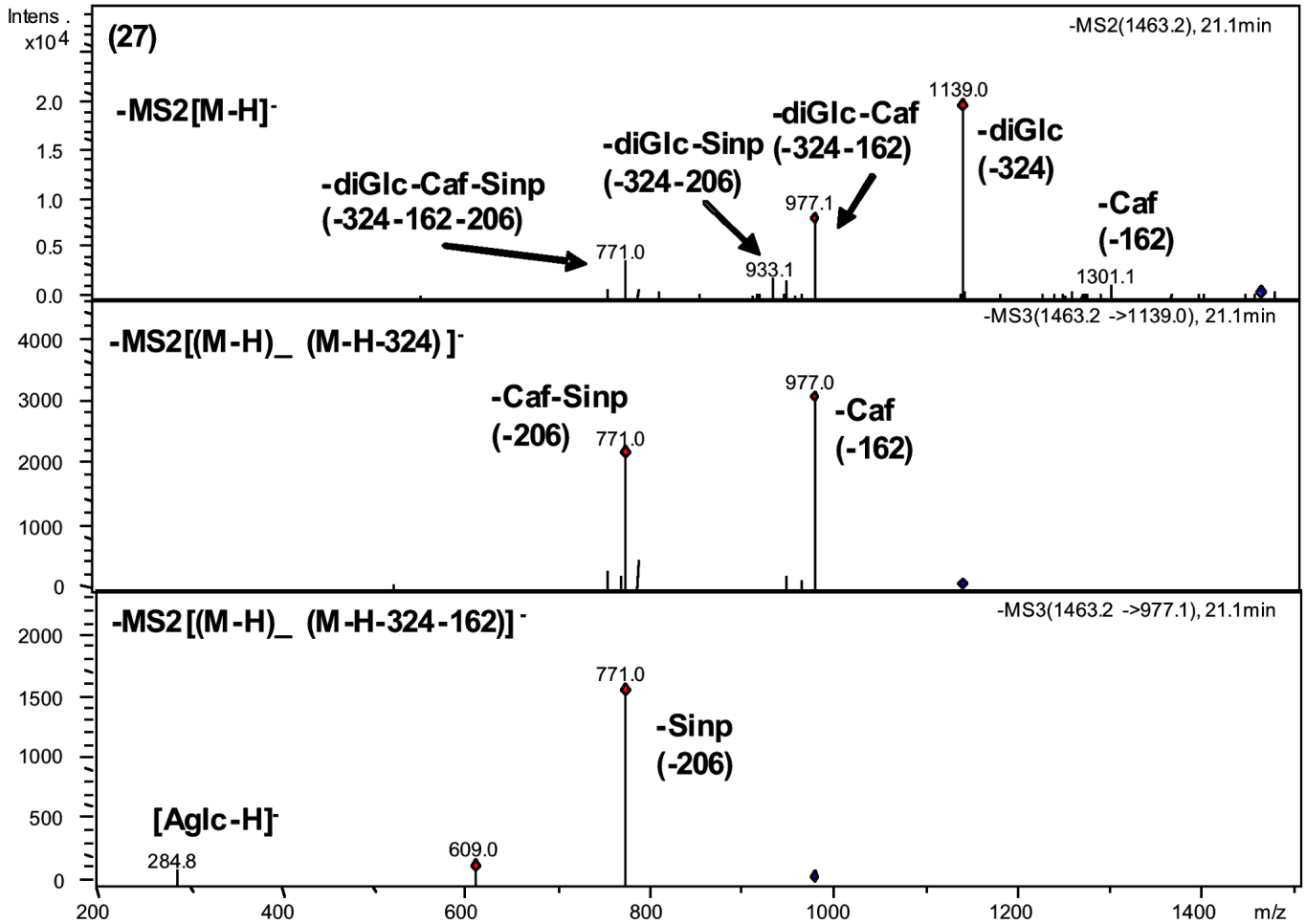

Fig. (2). Negative ion electrospray MS^2 mass spectrum of kaempferol-3-*O*-(caffeoyl/sinapoyl)-sophorotrioside-7-*O*-sophoroside (**27**). diGlc: diglucoside; Caf: caffeoyl; S: Sinp: sinapoyl.

O-(sinapoyl)-sophorotrioside (**15**), kaempferol-3-*O*-(methoxycaffeoyl)-sophorotrioside (**16**), kaempferol-3-*O*-(caffeoyl)-sophorotrioside (**20**), kaempferol-3-*O*-(sinapoyl)-sophorotrioside (**21**) and kaempferol-3-*O*-(feruloyl)-sophorotrioside (**22**).

In the reversed-phase HPLC analysis, the order of elution of the acylated derivatives, for the same type of glycosidic substitution, is not coincident with that of the free acids, as previously described [22]. Thus, the order of retention times of free acids is, from shorter to longer, caffeic < ferulic < sinapic, while in the studied acylated flavonoids the order is caffeoyl < sinapoyl < feruloyl. Another apparent difference, already reported [22], is that for acylated compounds without glycosylation at position 7 the corresponding deacylated compounds show similar or longer retention times.

Hydroxycinnamic Acyl Glycosides

In this group of compounds, synapoyl-glucoside (**8**) (*R*t 8.1 min; MS: $[M-H]^-$ 385, MS^2 $[M-H]^-$: 223) and several synapoyl-gentiobiosides (**13**, **19**, **25**, **30**, **31**, **33-37**) were characterized (Fig. **1A**, Table **5**). In the MS^2 fragmentation of the majority of them (**13**, **25**, **30**, **31**, **33-36**), the loss of 224 amu (sinapic acid) was observed to produce the base peak. Other ions that could be noticed, although in low abundance, corresponded to the deprotonated acids and the one due to the loss of 430 amu (-224-206). The fragmentation of compounds **19** and **37** does not follow the one previously indicated (Table **5**). These compounds were characterized as disinapoyl-gentiobioside (**13**), sinapoylcaffeoylferuloyl-gentiobioside (**19**), sinapoylmethoxycaffeoyl-gentiobioside (**25**), disinapoylmethoxycaffeoyl-gentiobioside (**30**), disinapoylcaffeoyl-gentiobioside (**31**), disinapoyl-gentiobioside isomer (**33**), sinapoylferuloyl-gentiobioside (**34**), trisinapoyl-gentiobioside (**35**), disinapoylferuloyl-gentiobioside (**36**) and sinapoyldiferuloyl-gentiobioside (**37**), being from the same type of those already described in broccoli [23, 25] and in tronchuda cabbage internal leaves [6] and seeds [5].

Thus, despite having found the same kind of compounds, the phenolic composition of *B. oleracea* var. *costata* shoots is distinct from that of the field grown plant parts. In fact, from the compounds characterized in the *in vitro* plant tissue, only 3-*p*-coumaroylquinic acid (**2**), kaempferol-3-*O*-(sinapoyl)-sophorotrioside-7-*O*-glucoside (**10**), 4-*p*-coumaroylquinic acid (**11**), kaempferol-3-*O*-(feruloyl)-sophorotrioside (**22**), kaempferol-3-O-sophoroside (**23**), disinapoyl-gentiobioside isomer (**33**), sinapoyl,feruloyl-gentiobioside (**34**), trisinapoyl-gentiobioside (**35**) and disinapoyl,feruloyl-gentiobioside (**36**) were described before in its external [3] or internal leaves [6] or seeds [5].

3.2. Antioxidant Capacity of Shoots

The antioxidant potential of *B. oleracea* var. *costata* shoots was evaluated against DPPH• and two reactive oxygen species (superoxide radical and hypochlorous acid). The DPPH• scavenging activity assay constitutes a screening method currently used to provide basic information on the antiradical activity of extracts. In the present study, the aqueous lyophilized extract of the shoots displayed a strong antioxidant capacity in a concentration-dependent manner, with an IC_{50} at 445 µg/mL (Fig. **3**).

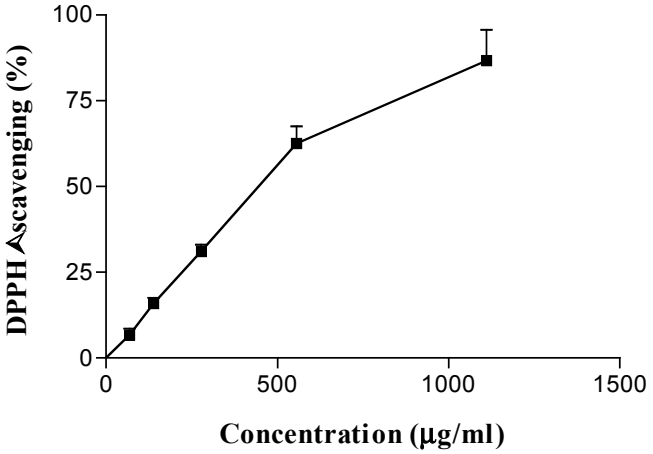

Fig. (3). Effect of *B. oleracea* var. *costata* shoots on DPPH• reduction. Values show mean ± SE from 3 experiments performed in triplicate.

Reactive oxygen species produced *in vivo* include superoxide radical, hydrogen peroxide and hypochlorous acid. Hydrogen peroxide and superoxide radical can interact in the presence of certain transition metal ions to yield the highly-reactive oxidizing species hydroxyl radical [26]. Superoxide radical scavenging activity was determined by monitoring the effect of *B. oleracea* var. *costata* shoots lyophilised extract on the reduction of NBT to the blue chromogen formazan. An effective concentration-dependent antioxidant capacity was found against superoxide radical generated in the NADH/PMS system, with an IC_{50} at 100 µg/mL (Fig. **4**).

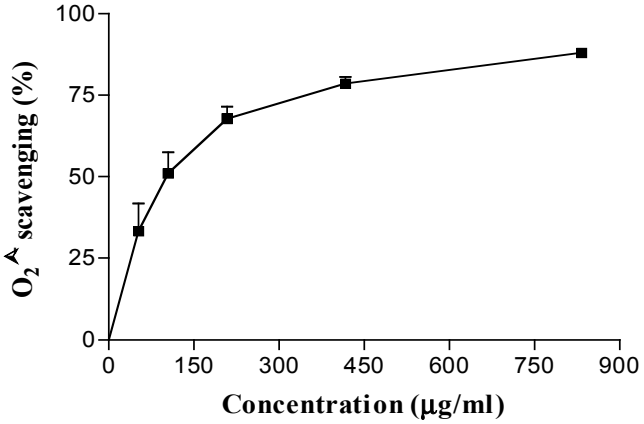

Fig. (4). Effect of *B. oleracea* var. *costata* shoots against superoxide radical generated in a chemical (NADH/PMS) system. Values show mean ± SE from 3 experiments performed in triplicate.

Hypochlorous acid can be produced *in vivo* by the peroxidation of Cl^- ions catalyzed by neutrophil-derived myeloperoxidase in the presence of H_2O_2 [26]. HOCl deleterious effects include cell lysis, activation of collagenase and gelatinase and inactivation of antioxidants enzymes like catalase [27, 28]. In this work, the oxidizing capacity of HOCl induced the oxidation of TNB (λ_{max} = 412 nm) to form DTNB (λ_{max} = 325 nm). Under the assayed conditions, the lyophilised extract of shoots exhibited a weak antioxidant protective activity against damage by HOCl, although it was concentration dependent (Fig. **5**).

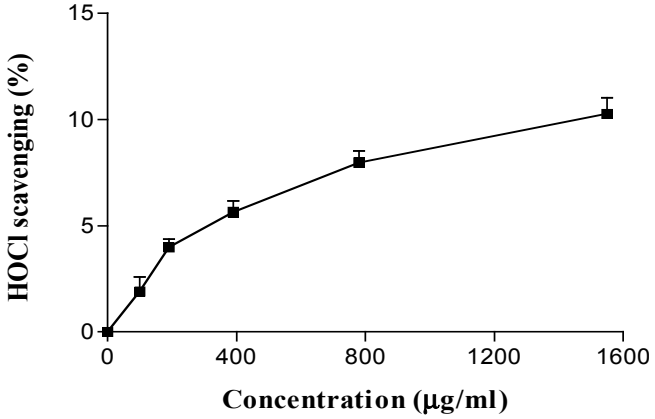

Fig. (5). Effect of *B. oleracea* var. *costata* shoots on the oxidation of TNB by HOCl. Values show mean ± SE from 3 experiments performed in triplicate.

According to the results obtained in these assays and in comparison with data from *B. oleracea* var. *costata* field grown plant tissue [5, 6, 8], it may be concluded that, in general terms, the shoots exhibit higher antioxidant potential than the leaves, but lower than the seeds. This is most probably related to the different phenolic compositions as referred above, since only a few of these compounds are common to all three systems. In addition, only the flavonol glycosides [29, 30] and hydroxycinnamic esters [31] have demonstrated antioxidant activities. The presence of several acylated flavonols, namely caffeoyl derivatives, must also be taken into account, since these compounds are known for their high scavenging ability due to the *o*-dihydroxy structure in the caffeoyl moiety, which confers great stability to the radical form and participates in the electron delocalization [29]. Finally, the complexity of the extract makes the isolation of these compounds difficult, and their chemical characteristics make their synthesis very challenging. Although no comparison with authentic standards could be performed because the characterized compounds are not commercially available, one may suggest that they contribute to some extent to the effects observed with the aqueous extract, which could be used as an antioxidant. Additionally, the activity exhibited by the whole extract corresponds to the resulting sum of the interactions of the several constituents.

4. CONCLUSIONS

The screening of the phenolic composition of *B. oleracea* var. *costata* shoots by LC-DAD-MS/MS revealed the existence of a high number of chlorogenic acids, kaempferol and quercetin derivatives, and hydroxycinnamic acyl glycosides, the majority of them distinct from those described previously in *in vivo* material. The results obtained are very promising, demonstrating that *in vitro* cultures of *B. oleracea* var. *costata*, namely its shoots, may constitute an alternative way

for production of complex phenolic antioxidative compounds. In addition, they also suggest that the inclusion of this material in the diet could be useful in the prevention of free radical-mediated diseases.

ACKNOWLEDGEMENT

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ABBREVIATIONS

BAP	=	Benzylaminopurine
GA	=	Gibberellic acid
LC-DAD-MS/MS	=	High-Performance Liquid Chromatography-diode-array detector–mass spectrometry
NAA	=	Naphthaleneacetic acid
*R*t	=	Retention time

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4.15. *In vitro* cultures of *Brassica oleracea* L. var. *costata* DC: Potential plant bioreactor for antioxidant phenolic compounds.

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In Vitro Cultures of *Brassica oleracea* L. var. *costata* DC: Potential Plant Bioreactor for Antioxidant Phenolic Compounds

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In this work were studied the phenolic composition of in vitro material (shoots, calli, and roots) of *Brassica oleracea* var. *costata* and its antioxidant capacity. Samples were obtained in different culture medium, with distinct supplementations to verify their influence on those parameters. Phenolic determination was achieved by HPLC-DAD. Antioxidant activity was assessed against DPPH•. In calli and roots no phenolic compound was identified. In shoots was verified the presence of 36 compounds, which included hydroxycinnamic acids, flavonoids (kaempferol and quercetin derivatives), and hydroxycinnamic acyl glycosides (with a predominance of synapoyl gentiobiosides). MS liquid medium supplemented with 2 mg/L benzylaminopurine (BAP) and 0.1 mg/L naphthaleneacetic acid (NAA) revealed to be the best in vitro condition to produce shoot material with highest phenolic compound contents and stronger antioxidant potential, thus with a possible increase of health benefits.

KEYWORDS: ***Brassica oleracea* L. var. *costata* DC; shoots; calli; roots; phenolic compounds; antioxidant activity**

INTRODUCTION

In recent years attention has been focused on plants as a source of phytochemicals with chemopreventive and chemotherapeutic potential. These phytochemicals comprise different structures and involve several protective mechanisms, plant secondary metabolites being the most likely candidates for health-promoting effects. Among those presenting antioxidant capacity, phenolic compounds constitute one of the most important groups (*1*). Several studies report that these compounds show a preventive effect against chronic diseases, such as cancer, atherosclerosis, nephritis, diabetes mellitus, rheumatism, Alzheimer's, Parkinson's, ischemic and cardiovascular diseases, which are associated with an excess of free radicals (*2, 3*).

The chemical composition of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) has already been studied, revealing this to be a good source of interesting compounds, such as organic acids, phenolic compounds, and amino acids. The different tronchuda cabbage vegetal materials exhibit distinct qualitative and quantitative compositions (*4*−*8*). In addition, all of these matrices have already displayed antioxidant capacity against different reactive species of both oxygen (*4, 5, 9*) and nitrogen (*10*). Thus, as it is widely consumed, this species constitutes a dietary source of phytochemicals useful to achieve optimal health.

In many cases, the chemical synthesis of these metabolites is not possible or economically feasible. The obtainment of these compounds in plant cultivation or from plants grown in nature is not always satisfactory, because they are exposed to different environmental, nutritional, and stress conditions, which can alter the quantitative and qualitative profile of compounds in plants (*11*). In vitro cultures are found to be an attractive option relative to the traditional methods of planting: growth conditions can be controlled, they allow a continuous and rapid propagation of plants, the production of specific metabolites can be stimulated, and a well-defined production system can result in higher yield and more consistent quality of the products (*12*). According to this, the large-scale plant cell and tissue cultures have been considered as a viable alternative source of phytochemicals. They can be regarded as a bioreactor, where reactions take place under optimum external environment to meet the needs of the biological system, so that a high yield of

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a bioprocess is achieved. Using plants as bioreactors allows valuable natural products to be obtained in a short period of time and space, with a maximum profitability. These products may be highly concentrated in interesting phytochemicals, which can be further used for human consumption or pharmaceutical industry (*13, 14*). Different media and hormonal supplementations can influence the production of biomass and of specific metabolites. The most important groups of hormonal supplements are auxins (such as naphthaleneacetic and dichlorophenoxyacetic acids) and cytokinins (such as benzylaminopurine), but gibberellins (gibberellic acid) are also often used (*12*). Generally, auxins are associated with cell growth and root initiation, cytokinins are responsible for cell division and shoot growth, and gibberellins influence stem elongation, leaf differentiation, and photomorphogenesis (*12, 15*). Phenolic composition can be modulated to achieve the best antioxidant activity (*16*).

In vitro cultures of *B. oleracea* var. *costata* would afford a good model system for studying the accumulation of antioxidant compounds. Several studies reported the in vitro development of vegetable materials from *Brassica* and some of them involved *B. oleracea* species (*17−21*). As far as we know, only one work described the presence of phenolic compounds in *B. oleracea* var. *costata* shoots (*22*). The chemical structures of the phenolics present are complex, which renders their synthesis almost impossible. In addition, studies concerning the effects of medium and hormonal supplementations on the production of phenolic compounds and antioxidant capacity of shoots and other in vitro materials are nonexistent.

The aim of this study was to evaluate the influence of different media and distinct supplementations on the phenolic composition and antioxidant potential of *B. oleracea* var. *costata* materials obtained from in vitro culture (roots, calli, and shoots). The phenolic profile was established by reversed-phase HPLC-DAD, and the antioxidant capacity was assessed against DPPH radical.

MATERIALS AND METHODS

Standards and Reagents. Chlorogenic, *p*-coumaric, ferulic, and sinapic acids, kaempferol-3-*O*-rutinoside, and quercetin-3-*O*-galactoside were purchased from Extrasynthése (Genay, France). DPPH was obtained from Sigma Chemical Co. (St. Louis, MO), methanol was purchased from Merck (Darmstadt, Germany), acetic acid was from Fisher Scientific (Leicestershire, U.K.), and hydrochloric acid was from Pancreac (Barcelona, Spain). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

Solid-Phase Extraction (SPE) Columns. The C18 non-end-capped (NEC) columns (50 μm particle size, 60 Å porosity; 10 g of sorbent mass/70 mL of reservoir volume) were obtained from Chromabond (Macherey-Nagel, Germany).

Plant Material and Sterilization. *B. oleracea* L. var. *costata* DC seeds were dipped in a 90% ethanol solution for 10 min, followed by washing with sterilized water, before surface sterilization with a 5% sodium hypochlorite solution for 10 min. After a rinse with sterilized water, seeds were germinated on autoclaved MS (*23*) basal medium devoid of growth regulators, with 20 g/L sucrose and solidified with 8 g/L agar. Seed germination was performed in a growth chamber in complete darkness for 5 days and then under 16 h light and 23 °C/8 h dark and 16 °C cycles.

In Vitro Cultures. Internodal shoot segments (ca. 10 mm), obtained from aseptic 3-week seedlings, were used as primary explants in the development of shoot, calli, and root cultures. Root cultures were established on MS liquid medium with no hormonal supplementation or supplemented with 1 μM gibberellic acid (GA). Calli cultures were established on MS solid medium supplemented with 1 and 2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4D) or on B5 solid medium supplemented with 2 mg/L benzylaminopurine (BAP) combined with 0.1 mg/L naphthaleneacetic acid (NAA). The shoot cultures were established on MS and B5 media with distinct supplementations (**Table 1**). The culture conditions were those described above and were subcultured in the same medium with intervals of 3 weeks. At the end of the third subculture period, shoots, calli, and roots were withdrawn and lyophilized for 7 days.

Table 1. Characterization of *Brassica oleracea* var. *costata* Shoot Sample

sample	medium	type of medium	supplementation
A	MS	liquid	
B	MS	liquid	1 μM GA + 2 mg/L BAP + 0.1 mg/L NAA
C	MS	liquid	2 mg/L BAP + 0.1 mg/L NAA
D	MS	solid	2 mg/L BAP + 0.1 mg/L NAA + 2 mg/L $AgNO_3$
E	MS	solid	1 mg/L 2,4D
F	MS	solid	2 mg/L 2,4D
G	MS	solid	2 mg/L BAP + 0.1 mg/L NAA
H	B5	liquid	2 mg/L BAP + 0.1 mg/L NAA
I	B5	solid	2 mg/L BAP + 0.1 mg/L NAA

Phenolic Compound Extraction. Each sample (ca. 0.1 g) was thoroughly mixed with methanol until complete extraction of the phenolic compounds (negative reaction to 20% NaOH) and then filtered through a Büchner funnel. The methanolic extract was concentrated to dryness under reduced pressure (40 °C) and redissolved in water acidified to pH 2 with HCl. The solution obtained was applied in the C18 (NEC) column, previously conditioned with 30 mL of methanol and 70 mL of acidified water. Polar compounds were removed with the aqueous solvent, and the retained phenolic compounds were then eluted with 50 mL of methanol. The extract was concentrated to dryness under reduced pressure (40 °C) and redissolved in methanol (1 mL).

HPLC-DAD Analysis of Phenolic Compounds. The separation was carried out with a HPLC unit (Gilson) and a 250 × 4.6 mm i.d., 5 μm Spherisorb ODS2 column (Waters, Milford, MA). The solvent system was a mixture of 1% acetic acid in water (A) and methanol (B), at a flow rate of 1 mL/min. Elution started with 20% B and reached 50% B at 30 min, 80% B at 37 min, and 100% B at 40 min. Detection was achieved with a Gilson diode array detector. Spectroscopic data from all peaks were recorded at 330 nm. The data were processed on Unipoint system software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities. The different phenolic compounds were identified by comparing their chromatographic behavior and UV−vis spectra in the 200−400 nm range with authentic standards and with published data (*22*).

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. The peaks in the chromatograms were integrated using a default baseline construction technique. Because standards of the compounds identified in the shoot methanolic extracts were not commercially available, the derivatives of caffeic, *p*-coumaric, ferulic, and sinapic acids were quantified as chlorogenic, *p*-coumaric, ferulic, and sinapic acids, respectively; kaempferol derivatives were quantified as kaempferol-3-*O*-rutinoside and quercetin derivatives as quercetin-3-*O*-galactoside. Ferulic, *p*-coumaric, and sinapic acids were quantified as themselves.

DPPH• Scavenging Activity. The antiradical activity of the extracts was determined spectrophotometrically in a Multiskan Ascent plate reader (Thermo Electron Corp.), by monitoring the disappearance of DPPH• at 515 nm, according to a described procedure (*5, 9*). The reaction mixture in the sample wells consisted of 25 μL of methanolic extract and 200 μL of methanolic solution of DPPH• 150 μM. The plate was incubated for 30 min at room temperature after the addition of DPPH•. Three experiments were performed in triplicate.

RESULTS AND DISCUSSION

Phenolics Composition. The analysis by HPLC-DAD of the methanolic extracts of *B. oleracea* var. *costata* shoots revealed the presence of several phenolic acids and flavonoid derivatives. Thirty-six compounds were identified (**Figure 1**), which included 6 chlorogenic acids, 3 free hydroxycinnamic acids, 8 hydroxycinnamic acid gentiobiosides, and 15 and 4 kaempferol

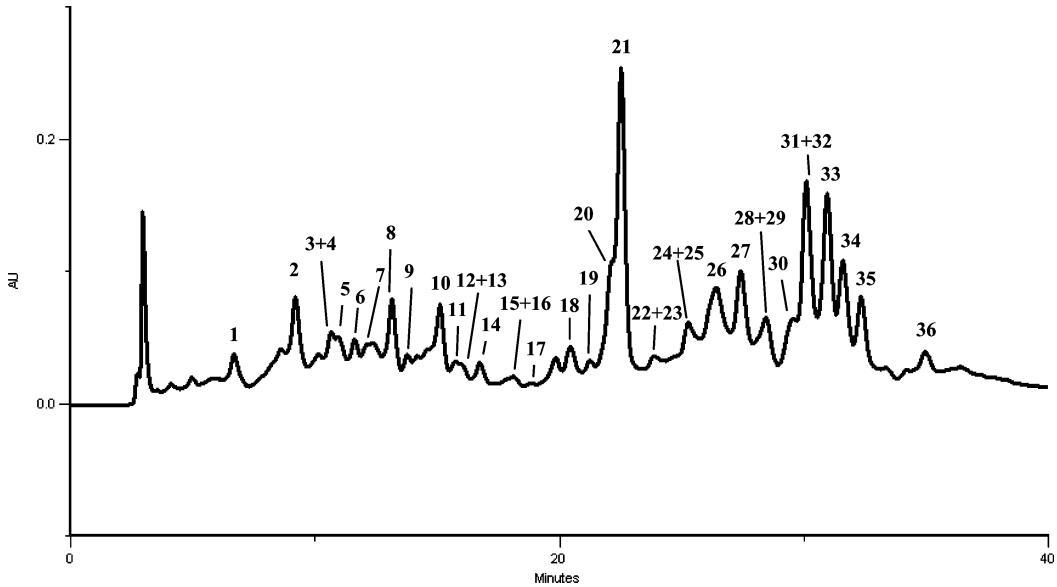

Figure 1. HPLC-DAD phenolics profile of *Brassica oleracea* var. *costata* shoots (sample C). Detection was at 330 nm. Peaks: (1) 3-caffeoylquinic acid; (2) 3-*p*-coumaroylquinic acid; (3) 3-feruloylquinic acid; (4) 4-caffeoylquinic acid; (5) kaempferol-3-*O*-(caffeoyl)-sophorotrioside-7-*O*-glucoside; (6) caffeoylferuloylquinic acid; (7) quercetin-3-*O*-(sinapoyl)sophorotrioside-7-*O*-glucoside; (8) kaempferol-3-*O*-(sinapoyl)sophorotrioside-7-*O*-sophoroside; (9) kaempferol-3-*O*-(sinapoyl)sophorotrioside-7-*O*-glucoside; (10) 4-*p*-coumaroylquinic acid; (11) kaempferol-3-*O*-(feruloyl)sophorotrioside-7-*O*-glucoside; (12) kaempferol-3-*O*-(sinapoyl)sophorotrioside-7-*O*-rhamnoside; (13) quercetin-3-*O*-(sinapoyl)sophorotrioside; (14) kaempferol-3-*O*-(methoxycaffeoyl)sophorotrioside; (15) kaempferol-3-*O*-(feruloyl)sophorotrioside-7-*O*-rhamnoside; (16) quercetin-3-*O*-sophoroside; (17) sinapoyl, caffeoyl, feruloyl-gentiobioside; (18) *p*-coumaric acid; (19) kaempferol-3-*O*-(caffeoyl)sophorotrioside; (20) ferulic acid; (21) sinapic acid; (22) kaempferol-3-*O*-(feruloyl)sophorotrioside; (23) kaempferol-3-*O*-sophoroside; (24) kaempferol-3-*O*-(methoxycaffeoyl/sinapoyl)sophorotrioside-7-*O*-sophoroside; (25) sinapoyl, methoxycaffeoyl-gentiobioside; (26) kaempferol-3-*O*-(methoxycaffeoyl/sinapoyl)sophorotrioside-7-*O*-glucoside; (27) kaempferol-3-*O*-(caffeoyl/sinapoyl)sophorotrioside-7-*O*-sophoroside; (28) kaempferol-3-*O*-(caffeoyl/sinapoyl)sophorotrioside-7-*O*-glucoside; (29) quercetin-3-*O*-(disinapoyl)sophorotrioside-7-*O*-glucoside; (30) disinapoyl, methoxycaffeoyl-gentiobioside; (31) disinapoyl, caffeoyl-gentiobioside; (32) kaempferol-3-*O*-(disinapoyl)sophorotrioside-7-*O*-glucoside; (33) 1-sinapoyl-2-feruloyl-gentiobioside; (34) 1,2,2′-trisinapoyl-gentiobioside; (35) 1,2′-disinapoyl-2-feruloyl-gentiobioside; (36) sinapoyl, diferuloyl-gentiobioside.

and quercetin derivatives, respectively. All of the detected compounds have already been reported in *B. oleracea* var. *costata* shoots (*22*), except *p*-coumaric, ferulic, and sinapic acids. However, qualitative and quantitative differences were noted between the phenolic profile of the several shoot samples, which were expected once they were obtained in different culture media, with distinct supplementations.

Data from the quantification of the identified compounds (**Table 2**) showed that MS liquid medium supplemented with 2 mg/L BAP and 0.1 mg/L NAA (sample C) allows the highest amount of phenolic compounds (ca. 19 g/kg) to be obtained. In a general way, the major compound in samples was 1-sinapoyl-2-feruloyl-gentiobioside (**33**), except for samples C, E, F, and G, in which the major compound was 1,2′-disinapoyl-2-feruloylgentiobioside (**35**).

When the media MS (samples C and G) and B5 (samples H and I), with the same hormonal supplementation (**Table 1**) were compared, higher production of phenolic compounds was observed in the MS medium (**Table 2**).

For liquid (samples C and H) and solid (samples G and I) media (**Table 1**) it was noted that liquid medium allows higher phenolic compound amounts (**Table 2**), which is not surprising; once in a liquid medium, there is higher nutrient availability, and their transport into the cells is easier (*24*). This occurs because in the liquid medium the absorption of nutrients can be made, theoretically, through all cellular surfaces, whereas in solid medium this absorption occurs only on the cells that directly contact the medium (*25*).

The addition of $AgNO_3$ (sample D) seems to lead to a decrease of phenolic production compared with the same medium without $AgNO_3$ (sample G) (**Tables 1** and **2**). This could be explained by the fact that $AgNO_3$ is an ethylene inhibitor, decreasing the levels of stress to which shoots were exposed and, consequently, the stimulation of phenolic compound accumulation (*26*).

The addition of GA (sample B) also seems to cause a decrease of the phenolic contents compared with the same medium without GA (sample C) (**Tables 1** and **2**). The hormone regulators can modulate key enzymes of phenolic biosynthesis pathways (*27*). In addition, the association of different hormone regulators can result in an antagonist or synergic response (*28*), which is a hypothesis to explain the observed decrease.

Two different concentrations of 2,4D were tested (samples E and F, **Table 1**), and it was verified that a higher phenolics production occurred in sample F, which corresponded to the addition of a higher concentration (2 mg/L) of this hormone regulator (**Table 2**). This finding is in accordance with a previous work in tobacco cells reporting the decrease of phenolic concentration with low level of 2,4D (*29*).

Independently of the shoot culture conditions, phenolic acid derivatives were the main compounds, varying between 77 and 93% of total phenolics (**Figure 2**). The synthesis of flavonoids was not preferred during shoot growth once the available nutrients are required for the primary metabolism, and mainly phenolic acids were synthesized (*7*). The sample supplemented with 2 mg/L BAP, 0.1 mg/L NAA, and 2 mg/L $AgNO_3$ (sample D) presents the higher flavonoid derivative relative amounts (23.7%), whereas sample H exhibits the lowest one (7.3%) (**Figure 2**).

By comparison of the media supplemented with different concentrations of 2,4D (samples E and F, **Table 1**) it was verified that the highest production of flavonoid derivatives occurred for higher supplementation of this growth regulator (sample F, **Figure 2**). The effect of 2,4D on some enzymes of

Table 2. Phenolic Composition of *Brassica oleracea* var. *costata* Shoots (Milligrams per Kilogram, Dry Basis)[a]

	sample[b]								
compound[c]	A	B	C	D	E	F	G	H	I
1			67.8 (0.4)	151.1 (1.1)	6.8 (1.6)	38.0 (0.5)	30.4 (0.7)		
2	2.4 (0.1)		95.0 (2.3)	62.5 (1.8)	19.5 (2.3)	43.9 (2.5)	28.2 (0.2)	2.8 (0.2)	2.6 (0.0)
3 + 4			115.8 (3.0)	80.0 (1.4)					
5	7.1 (0.7)		187.1 (0.8)						
6			126.5 (4.5)		35.6 (4.4)				
7			205.1 (6.3)	447.3 (18.4)			309.1 (35.7)		
8	98.7 (3.5)	47.5 (7.9)	304.6 (14.4)	342.2 (32.7)	80.3 (2.8)	537.7 (69.8)	418.8 (21.4)		
9	9.0 (0.5)	9.2 (0.5)	102.8 (0.6)	81.5 (3.5)			52.6 (0.2)		
10		30.7 (2.4)	106.6 (2.4)	24.8 (9.1)		28.1 (1.4)	2.3 (0.0)		
11			144.2 (0.1)						
12 + 13			157.5 (7.8)	11.8 (9.6)					
14	8.1 (0.5)	17.5 (0.2)	81.0 (3.3)						
15 + 16			42.3 (0.8)						
17			nq[d]						
18	3.9 (0.6)	1.2 (0.0)	30.4 (4.9)	2.3 (0.4)	2.5 (0.1)	4.4 (0.1)		2.1 (0.4)	2.7 (0.0)
19			5.9 (0.4)						
20	131.5 (7.6)	188.3 (2.1)	1617.2 (146.5)	462.1 (44.0)	390.5 (15.0)	539.3 (47.1)	217.3 (19.0)	61.3 (3.6)	186.9 (37.7)
21	254.4 (14.5)	257.1 (18.8)	2206.5 (209.2)	351.14 (42.8)	394.4 (28.1)	884.0 (23.1)	158.1 (1.4)	282.1 (25.2)	243.2 (10.9)
22 + 23	nq		71.8 (1.4)		6.0 (0.0)		32.9 (6.0)		
24 + 25			364.6 (16.5)	20.4 (14.5)	7.4 (1.0)	70.6 (0.7)	42.7 (4.2)		
26			694.4 (3.7)	290.4 (39.4)	44.8 (0.7)	230.6 (12.4)	144.2 (16.0)		
27			51.5 (2.8)		5.6 (0.0)	19.5 (0.5)	18.2 (0.2)		
28 + 29			330.3 (19.1)		44.2 (0.8)	198.0 (18.1)	108.2 (2.9)		
30			1051.5 (18.8)						
31 + 32	405.5 (5.8)	284.3 (17.4)	895.6 (9.9)	733.9 (25.4)	341.5 (8.3)	822.6 (60.2)	464.9 (55.2)	207.2 (4.4)	235.1 (5.1)
33	2493.3 (205.9)	2280.5 (110.1)	3213.5 (50.2)	1978.9 (151.6)	626.4 (29.2)	1226.5 (82.6)	843.4 (93.8)	1133.9 (123.2)	1026.6 (268.5)
34	186.0 (8.4)	243.5 (1.3)	1981.3 (31.1)	979.7 (68.6)	1052.1 (31.9)	3069.3 (48.7)	807.5 (71.1)	64.7 (1.4)	
35	584.8 (2.0)		3677.4 (45.8)	1619.6 (151.7)	1081.2 (156.8)	3340.9 (26.2)	2411.0 (110.6)	226.7 (13.8)	
36			1083.8 (30.8)	519.4 (25.9)	285.1 (85.6)	764.9 (14.9)	1812.0 (95.7)	850.2 (25.6)	
total	4184.9	3360.0	18969.5	8159.3	4423.7	11818.3	8905.0	2831.1	1697.0

[a] Results are expressed as means (standard deviation) of three determinations. [b] Sample characterization as in **Table 1**. [c] Compound numbers refer to peak numbers in **Figure 1**. [d] nq, not quantified.

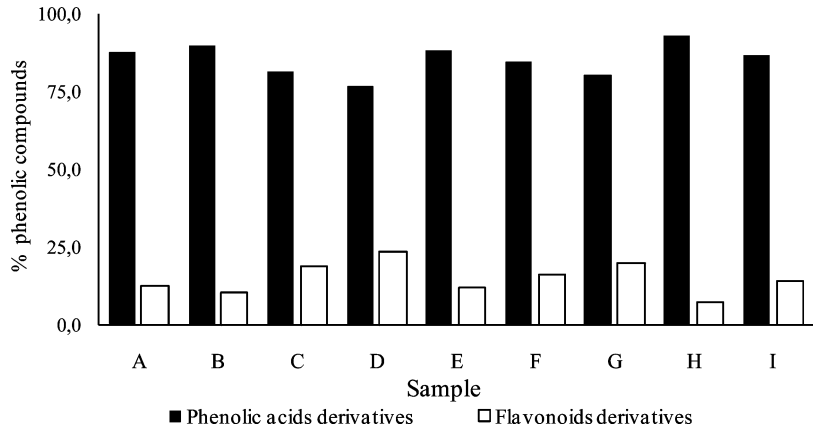

Figure 2. Relationship between phenolic acids and flavonoid derivatives in *Brassica oleracea* var. *costata* shoot samples. Characterization of samples was as in **Table 1**.

the flavonoid pathway, such as phenylalanine ammonia-lyase (PAL), was already reported. In studies with tobacco cells it was verified that PAL activity decreased for lower concentrations of 2,4D (*29−31*).

When comparing samples B and C (**Table 1**; **Figure 2**) we can see that the addition of GA to the medium supplemented with 2 mg/L BAP and 0.1 mg/L NAA caused a decrease of flavonoid derivatives. Previous works suggested that GA might inhibit enzymes involved in the biosynthesis of flavonoids, namely chalcone synthase, which may justify the decrease in flavonoid derivatives' production (*32*).

By comparison of the results obtained with those found for *B. oleracea* var. *costata* leaves obtained from in vivo cultures (*5*) it can be noted that the material produced in vitro presents a higher amount of phenolic compounds, independent of the medium and supplementation used.

In *B. oleracea* var. *costata* root and calli samples it was not possible to identify any phenolic compound. The lack of induction of phenolic metabolism in the roots may be explained by the fact that the available nutrients are being oriented for the primary metabolism (*33*). With respect to calli, this material may not express some genes responsible for the synthesis of certain secondary metabolites, such as phenolic compounds, because it is dependent upon plant differentiation: only in stages of greater differentiation are these promoters present, allowing the production of phenolics (*34*).

Antioxidant Activity. The DPPH$^{\bullet}$ assay provides basic information on the antiradical activity of extracts (*35*). The

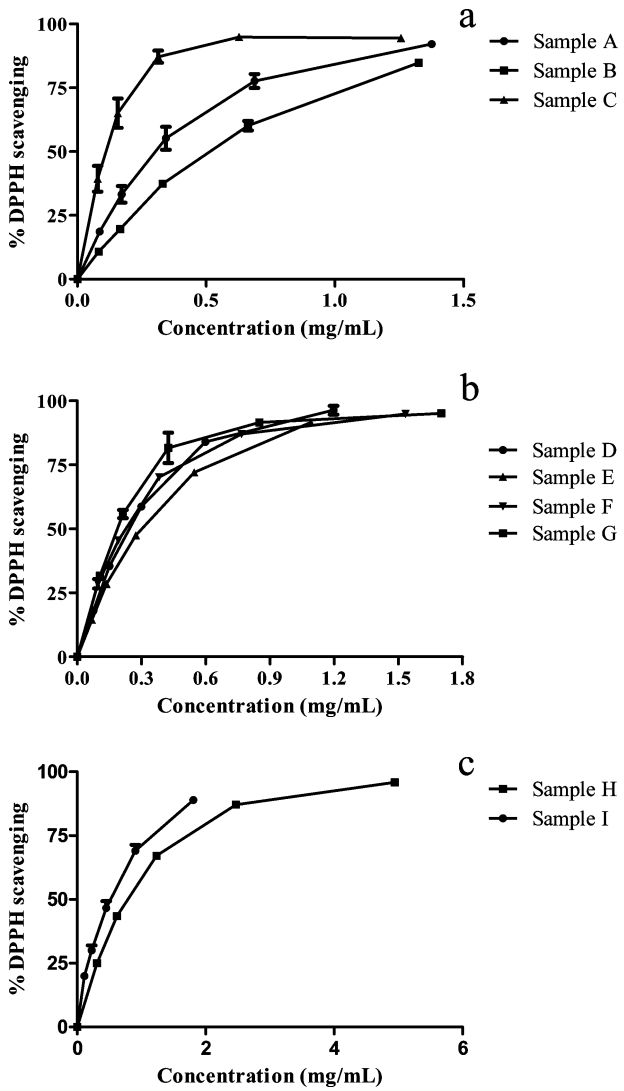

Figure 3. DPPH scavenging ability of methanolic extracts of *Brassica oleracea* var. *costata* shoots obtained on (**a**) liquid MS medium, (**b**) solid MS medium, and (**c**) B5 medium. Values show mean ± SE from three experiments performed in triplicate. Characterization of samples as in **Table 1**.

antioxidant activity was appraised in *B. oleracea* var. *costata* shoots because only this plant material presented phenolics. All of the analyzed shoot samples' methanolic extracts displayed a strong concentration-dependent antioxidant potential. The results were grouped by taking into account the medium characteristics (**Figure 3**).

Considering liquid MS medium (**Table 1**), supplementation with 2 mg/L BAP and 0.1 mg/L NAA (sample C) allowed us to obtain the in vitro material with the highest antioxidant activity ($EC_{50} = 0.111$ mg/mL). The shoots obtained on medium supplemented with GA (sample B) show less activity ($EC_{50} = 0.516$ mg/mL) than the medium without any supplementation (sample A; $EC_{50} = 0.303$ mg/mL) (**Figure 3**).

With regard to solid MS medium (**Table 1**), as for the liquid one, the shoots obtained on medium supplemented with 2 mg/L BAP and 0.1 mg/L NAA (sample G) exhibited the highest antioxidant activity ($EC_{50} = 0.136$ mg/mL), whereas supplementation with 1 mg/L 2,4D (sample E) led to the lowest one ($EC_{50} = 0.301$ mg/mL) (**Figure 3**).

By comparison of B5 media (**Table 1**), the sample obtained on the solid medium (sample I) exhibited higher antioxidant capacity ($EC_{50} = 0.520$ mg/mL) than the one developed on liquid medium (sample H, $EC_{50} = 0.789$ mg/mL) (**Figure 3**).

The observed antioxidant capacity can be related, at least in part, to shoot phenolic composition, once previous studies involving *B. oleracea* var. *costata* in vivo cultures and containing the same type of compounds (*5, 6, 9*) revealed these effects. In fact, in a general way, samples with higher phenolic content displayed the strongest antiradical ability, whereas those with lower levels of these compounds were less effective. In addition, *B. oleracea* var. *costata* in vitro material exhibited higher antioxidant potential than the one produced in vivo (*5*).

By analyzing all of the results it can be observed that MS liquid medium supplemented with 2 mg/L BAP and 0.1 mg/L NAA (sample C, **Table 1**) is the one which allows *B. oleracea* var. *costata* shoots with the highest antioxidant activity (**Figure 3**) and highest phenolic amount (**Table 2**) to be obtained. Thus, this medium can be considered to be the most appropriate for the production of *B. oleracea* var. *costata* shoots when it is intended to obtain material with these characteristics.

In conclusion, this work suggests that *B. oleracea* var. *costata* shoots may bring health benefits once they constitute a potential source of valuable secondary metabolites, such as antioxidant phenolic compounds. This species is considered to be a possible plant bioreactor to produce those metabolites, and shoots obtained in MS liquid medium supplemented with 2 mg/L BAP and 0.1 mg/L NAA seem to be the best option. This perspective deserves to be explored by food and pharmaceutical industries to obtain material that can be used either as nutraceuticals, food additives, or antioxidant supplements.

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4.16. Tronchuda cabbage flavonoids uptake by *Pieris brassicae*

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Tronchuda cabbage flavonoids uptake by *Pieris brassicae*

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Abstract

The flavonoid pattern of larvae of cabbage white butterfly (*Pieris brassicae* L.; Lepidoptera: Pieridae) reared on the leaves of tronchuda cabbage was analysed by HPLC-DAD-MS/MS-ESI. Twenty flavonoids were identified or characterised, namely 16 kaempferol and four quercetin derivatives. Kaempferol 3-*O*-sophoroside, a minor component of tronchuda cabbage, was found to be the main component in *P. brassicae* (15.8%). Apart from this, only two other flavonoids present in significant amounts in tronchuda cabbage (kaempferol 3-*O*-sophoroside-7-*O*-glucoside and kaempferol 3-*O*-sophoroside-7-*O*-sophoroside) were found in the larvae. The larvae have high amounts of quercetin derivatives (18.5%), which were present only in trace amounts in tronchuda cabbage extracts, suggesting that *P. brassicae* is able to selectively sequester these flavonoids. The occurrence of a high content of flavonoids not detectable in tronchuda cabbage extracts indicates that *P. brassicae* larvae are able to metabolize dietary flavonoids.
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Keywords: *Pieris brassicae* L. larvae; Tronchuda cabbage; *Brassica oleracea* L. var. *costata* DC; Flavonoids

1. Introduction

Larvae of *Pieris brassicae* L. (Lepidoptera: Pieridae) are specialists on crucifers, whereas adults feed of the nectar of a variety of plants. The larvae can feed on various species of Brassicaceae, namely, cauliflower, cabbage, turnip, nasturtium and, more rarely, on red cabbage and radish.

The close association between *Pieris* sp. butterflies and crucifers was linked to the presence of glucosinolates in these plants (Renwick, 2002). As well as glucosinolates, flavonoids can modulate the feeding behaviour of larvae and oviposition of adult insects (van Loon et al., 2002).

Flavonoid uptake is relatively widespread in the Lepidoptera, in particular in butterfly families like the Papilionidae, Nymphalidae, and Lycaenidae, where they form part of the wing pigmentation (Burghardt et al., 1997, 2001; Schittko et al., 1999). In fact, although most of pigments are synthesised de novo during scale development in the pupa, some are secondary plant metabolites taken up from the larval diet since insects are unable to synthesize flavonoids or their precursors de novo (Knüttel and Fiedler, 2001). Feeding experiments proved the dietary origin of the flavonoids (Burghardt et al., 1997; Schittko et al., 1999; Knüttel and Fiedler, 2001; Harborne and Grayer, 1994). Thus, flavonoid uptake and metabolism by insects is strongly dependent on the specific flavonoid pattern of their host plants (Burghardt et al., 1997, 2001; Schittko et al., 1999; Geuder et al., 1997).

The flavonoids sequestered by the larvae are subsequently metabolised, stored and transferred into the wings during the late pupal stage (Geuder et al., 1997). Flavonoids are known for their antioxidant potential (Ferreres et al., 2006; Vrchovská et al., 2006) and perhaps they act as antibiotic and antiviral agents in insects (Harborne and Grayer, 1994).

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In what concerns flavonoids patterns it was shown that (i) larvae sequester only specific fractions of the flavonoid load of the host plant, (ii) the sequestered flavonoids are subjected to various glycosydation processes, (iii) the flavonoid content of butterflies from the same species may drastically vary according to the host plant actually used during the larval stages, and (iv) female butterflies tend to be richer in flavonoids than males (Burghardt et al., 2001).

As referred above, flavonoids in the insects are positively associated with the amounts of flavonoids in the food that they had consumed (Burghardt et al., 2000). Larvae selectively sequester and metabolise quercetin and kaempferol derivatives, the predominant flavonoids in the analysed plants. Other flavonoids such as myricetin derivatives, flavones and isoflavonoids were mostly excreted (Burghardt et al., 2001).

As far as we know, there is no study concerning the sequestration of phenolic compounds by *P. brassicae* from tronchuda cabbage leaves (*Brassica oleracea* L. var. *costata* DC) or other cabbages. This study can be relevant from the nutritional point of view, considering that the larvae may accumulate or even metabolize trochuda cabbage constituents, namely complex flavonol glycosides (Ferreres et al., 2005, 2006), constituting a source of potential bioactive compounds not available in nature.

In this paper, the flavonoids present in larvae of *P. brassicae* were tentatively identified by HPLC-DAD-MS/MS-ESI and they were compared to the flavonoid pattern of the tronchuda cabbage external leaves, which were the only ones on which they have fed. Some considerations about ingestion, metabolism and accumulation of flavonoids in *P. brassicae* are made.

2. Results and discussion

2.1. Characterisation of P. brassicae phenolic compounds

The HPLC-DAD-MS/MS-ESI screening of the aqueous methanolic extract of *P. brassicae* larvae (Fig. 1) shows several peaks with UV spectra characteristic of flavonols, with two maxima at around 260 and 350 nm (Table 1), and their corresponding acylated derivatives (Table 2). The UV spectra shape of the acylated flavonols resembles the overlapping of a flavonol spectrum with a hydroxycinnamic acid one, with a broad maximum around 310–330 nm and a short maximum or shoulder around 265–271 nm (Table 2), which, therefore, can be misunderstood as a cinnamic acid derivative. Some spectra are poorly defined because the compounds are found in trace amounts and/or coelute with other ones. Thus, UV spectra of compounds **7**, **10** and **12** are not included in Table 2, and those of compounds **8**, **13** and **16** differ from the expected for isolated and purified compounds, probably due to possible co-elution with other products.

The ESI-MS/MS ion trap study confirms the above mentioned and shows several flavonoids which are structurally similar to those already described in various Brassicaceae species (Ferreres et al., 2005, 2006; Llorach et al., 2003; Vallejo et al., 2004). These compounds are characterised to be

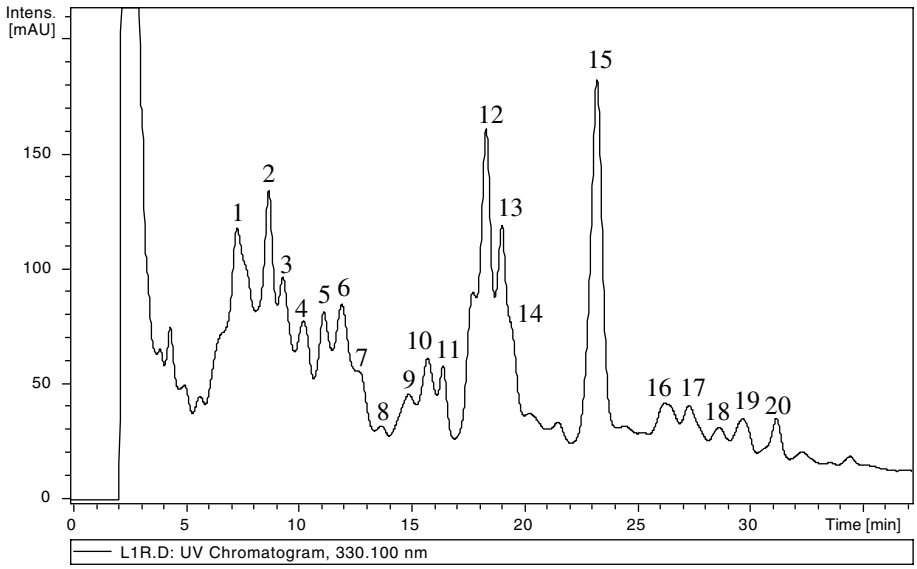

Fig. 1. HPLC-DAD phenolic profile of larvae hydromethanolic extract. Detection at 330 nm. Peaks: (**1**) quercetin 3-*O*-sophoroside-7-*O*-glucoside; (**2**) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (**3**) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside; (**4**) quercetin 3-*O*-(feruloyl)-triglucoside-7-*O*-glucoside; (**5**) kaempferol 3-*O*-(sinapoyl)-triglucoside-7-*O*-glucoside; (**6**) kaempferol 3-*O*-(feruloyl)-triglucoside-7-*O*-glucoside; (**7**) kaempferol 3-*O*-(*p*-coumaroyl)-triglucoside-7-*O*-glucoside; (**8**) kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside-7-*O*-glucoside; (**9**) kaempferol 3-*O*-(caffeoyl)-sophoroside-7-*O*-glucoside; (**10**) quercetin 3-*O*-(*p*-coumaroyl)-sophoroside; (**11**) kaempferol 3-*O*-(*p*-coumaroyl)-triglucoside; (**12**) kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside; (**13**) kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside; (**14**) quercetin 3-*O*-sophoroside; (**15**) kaempferol 3-*O*-sophoroside; (**16**) kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside (isomer); (**17**) kaempferol 3-*O*-(disinapoyl)-triglucoside-7-*O*-glucoside; (**18**) kaempferol 3-*O*-(feruloyl/sinapoyl)-triglucoside-7-*O*-glucoside; (**19**) quercetin 3-*O*-(feruloyl)-triglucoside; (**20**) kaempferol 3-*O*-glucoside.

Table 1
R_t, UV, -MS: $[M-H]^-$, -$MS^2[M-H]^-$ and -$MS^3[(M-H) \rightarrow 625/609]^-$ data of flavonol glycosides without acylation found in *P. brassicae*[a]

Compounds[b]	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	-$MS^2[M-H]^-$ (*m/z*) (%)			-$MS^3[(M-H) \rightarrow 625/609]^-$ (*m/z*) (%)		
				−162	−180	−324	−162	−180	−324
1 Querc-3-Soph-7-Glc[c]	7.2		787	625 (100)			463 (25)	445 (50)	300 (100)
2 Kaempf-3-Soph-7-Glc	8.6	267, 347	771	609 (100)			447 (10)	429 (35)	285 (100)
3 Kaempf-3-Soph-7-Soph[c]	9.2		933	771 (25)		609 (100)		429 (45)	285 (100)
14 Querc-3-Soph	19.4	255, 267, 350	625	463 (15)	445 (25)	300 (100)			
15 Kaempf-3-Soph	23.1	265, 293sh, 347	609	447 (10)	529 (50)	285 (100)			
20 Kaempf-3-Glc	31.0	265, 295sh, 348	447	285 (100)					

[a] Main observed fragments. Other ions were found but they have not been included.
[b] Querc, quercetin; Kaempf, kaempferol; Soph, sophoroside; Glc, glucoside.
[c] Compounds in trace amounts and hidden by other. Their UV spectra have not been observed properly.

Table 2
R_t, UV, -MS: $[M-H]^-$, -$MS^2[M-H]^-$ and -$MS^3[(M-H) \rightarrow (M-H-162)]^-$ data of flavonol acyl-glycosides found in *P. brassicae*[a]

Compounds[b]	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	Flavonol-3(acyl)glycosyl-7-glucoside derivatives					
				$MS^2[M-H]^-$	$MS^3[(M-H) \rightarrow (M-H-162)]^-$				
				-Glc (162)	-Caf (162)	-MCaf (192)	-*p*Coum (146)	-Fer (176)	-Sinp (206)
4 Querc-3-(Fer)triGlc-7-Glc	10.1	265, 291sh, 321	1125	963				787	
5 Kaempf-3-(Sinp)triGlc-7-Glc	11.1	269, 290sh, 325	1139	977					771
6 Kaempf-3-(Fer)triGlc-7-Glc	11.8	270, 299sh, 323	1109	947				771	
7 Kaempf-3-(*p*-Coum)triGlc-7-Glc[c]	12.5		1079	917			771		
8 Kaempf-3-(MCaf)Soph-7-Glc	13.6	271, 325	963	801		609			
9 Kaempf-3-(Caf)Soph-7-Glc	14.8	269, 320	933	771	609				
17 Kaempf-3-(diSinp)triGlc-7-Glc[d]	27.2	270, 331	1345	1183					977[d]
18 Kaempf-3-(FerSinp)triGlc-7-Glc[e]	28.6	271, 331	1315	1153				977	947[e]
				Flavonol-3(acyl)glycoside derivatives					
					$MS^2[M-H]^-$				
					-Caf	-MCaf	-*p*Coum	-Fer	-Sinp
10 Querc-3-(*p*-Coum)Soph[c]	15.8		771				625		
11 Kaempf-3-(*p*-Coum)triGlc	16.2	270sh, 293sh, 315	917				771		
12 Kaempf-3-(*p*-Coum)Soph[c]	18.2		755				609		
13 Kaempf-3-(MCaf)Soph	18.9	269, 325	801			609			
16 Kaempf-3-(*p*-Coum)Soph (isom)	26.1	267, 338	755				609		
19 Querc-3-(Fer)triGlc	29.5	269, 327	963					787	

[a] Main observed fragments. Other ions were found but they have not been included.
[b] Querc, quercetin; Kaempf, kaempferol; Soph, sophoroside; Glc, glucoside; Fer, feruloyl; Sinp, sinpapoyl; *p*-Coum, *p*-coumaroyl; Caf, caffeoyl; MCaf, methoxycaffeoyl.
[c] Compounds in trace amounts and hidden by other. Their UV spectra have not been observed properly.
[d] $MS^4(1345 \rightarrow 1183 \rightarrow 977)$: 771.
[e] $MS^4(1315 \rightarrow 1153 \rightarrow 947)$:771.

kaempferol and quercetin derivatives, the later in lesser amounts, with a high degree of glycosilation, as well as a possible acylation at the glycosidic fraction linked to the hydroxyl group at the 3 position of the flavonol.

From the MS study it is possible to distinguish a group of flavonol glycoside derivatives without acylation (Table 1), whose MS fragmentations (Ferreres et al., 2004) are characteristic of flavonol-3-*O*-sophoroside-7-*O*-glucoside (compounds **1** and **2**) (Fig. 2), flavonol-3-*O*-sophoroside-7-*O*-sophoroside (**3**), flavonol-3-*O*-sophoroside (**14** and **15**) and flavonol-3-*O*-glucoside (**20**).

On the other hand, the MS fragmentation pattern of the acylated flavonol derivatives (Vallejo et al., 2004) confirms the presence of a series of flavonol-3-*O*-(acyl)glycoside-7-*O*-glucoside (Table 2) (compounds **4**–**9**, **17** and **18**), in which we can observe a loss of 162 u in the $MS^2[M-H]^-$ due to the break of glucose at the 7-position. The $MS^3[(M-H) \rightarrow (M-H-162)]^-$ shows the loss of the acyl radical giving the aglycone fragment linked to the glycosidic fraction at the 3-position, triglucoside (**4**–**7**) or sophorose (**8** and **9**) (Table 2). For the diacylated derivatives, compounds **17** and **18**, a new MS event

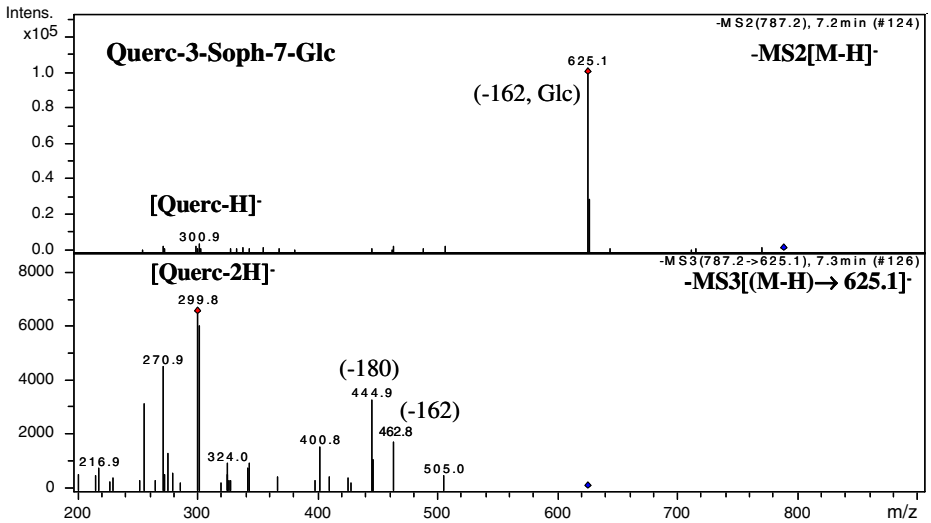

Fig. 2. MS^n analysis of quercetin 3-*O*-sophoroside-7-*O*-glucoside (**1**).

($MS^3[(M-H) \rightarrow (M-H\text{-}162) \rightarrow (M-H\text{-}162\text{-acyl})]^-$) was necessary to obtain the referred fragment.

Another set of complex flavonol derivatives (compounds **10**–**13**, **16** and **19**) shows a MS fragmentation (Table 2) in which $MS^2[M-H]^-$ event the loss could be interpreted as that of an acyl radical to give rise to the aglycone fragment linked to the glycosidic fraction at the 3-position (Figs. 3 and 4), confirming the lack of glycosilation at the 7-position (Vallejo et al., 2004). In this group of compounds it is observed an apparently anomalous chromatographic behaviour of compounds **10**, **12** and **13**, once they elute before the deacylated compounds from which they derive from. This behaviour has already been described for similar compounds found in cauliflower (Llorach et al., 2003), being noticed that the acylated derivatives at the 3-position of the sugar and without glycosilation in the 7-position exhibited an apparent irregular retention time. On the other hand, the presence of two isomers of kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside with lower (compound **12**) and higher retention times (compound **16**) than kaempferol 3-*O*-sophoroside (**15**) indicates that the position of the acylation over the sugar also influences the order of elution.

In addition, the loss of 146 u in the MS fragmentation of compounds **7**, **10**, **11**, **12** and **16**, the majority of them with poor or badly defined UV spectrum as mentioned above, could be due to the presence of a rhamnosyl radical, instead of a *p*-coumaroyl one. However, in tronchuda cabbage leaves neither rhamnosyl nor *p*-coumaroyl derivatives were found (Ferreres et al., 2005, 2006). So, we consider that the demethoxylation of the sinapoyl and/or feruloyl derivatives originating *p*-coumaroyl derivatives is more likely to occur during the metabolism process in the larvae, as indicated below, rather than the demethylation of glucose to give rise to rhamnosyl derivatives.

Thus, in *P. brassicae* the following compounds were identified or tentatively identified: quercetin 3-*O*-sophoroside-7-*O*-glucoside (**1**), kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**2**), kaempferol 3-*O*-sophoroside-7-*O*-sophoroside (**3**), querce-

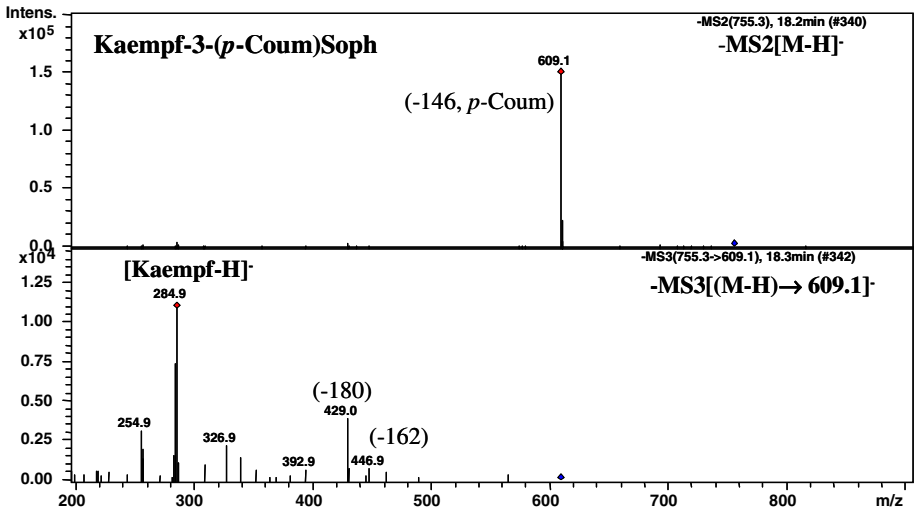

Fig. 3. MS^n analysis of kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside (**12**).

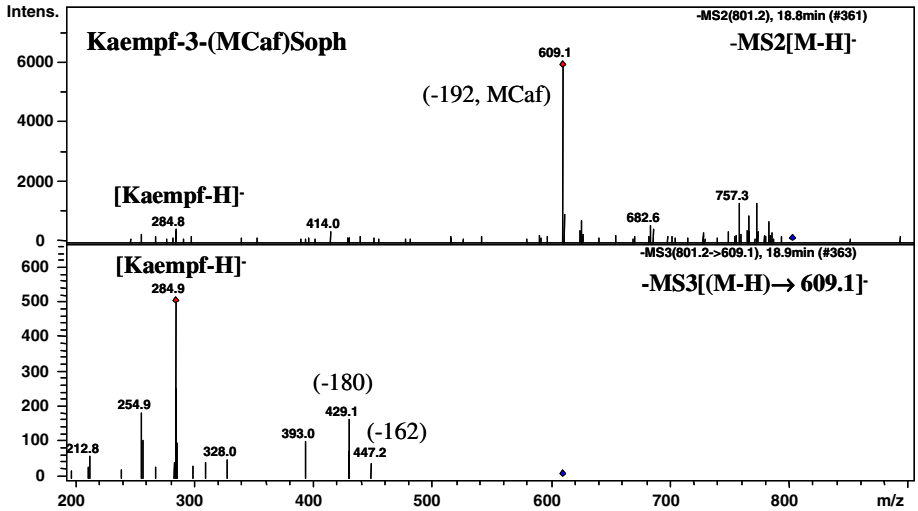

Fig. 4. MS^n analysis of kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside (**13**).

tin 3-*O*-(feruloyl)-triglucoside-7-*O*-glucoside (**4**), kaempferol 3-*O*-(sinapoyl)-triglucoside-7-*O*-glucoside (**5**), kaempferol 3-*O*-(feruloyl)-triglucoside-7-*O*-glucoside (**6**), kaempferol 3-*O*-(*p*-coumaroyl)-triglucoside-7-*O*-glucoside (**7**) (tentatively), kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside-7-*O*-glucoside (**8**) (tentatively), kaempferol 3-*O*-(caffeoyl)-sophoroside-7-*O*-glucoside (**9**) (tentatively), quercetin 3-*O*-(*p*-coumaroyl)-sophoroside (**10**) (tentatively), kaempferol 3-*O*-(*p*-coumaroyl)-triglucoside (**11**) (tentatively), two isomers of kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside (**12** and **16**) (tentatively), kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside (**13**) (tentatively), quercetin 3-*O*-sophoroside (**14**), kaempferol 3-*O*-sophoroside (**15**), kaempferol 3-*O*-(disinapoyl)-triglucoside-7-*O*-glucoside (**17**), kaempferol 3-*O*-(feruloyl/sinapoyl)-triglucoside-7-*O*-glucoside (**18**), quercetin 3-*O*-(feruloyl)-triglucoside (**19**) and kaempferol 3-*O*-glucoside (**20**).

2.2. Comparison between flavonoids in P. brassicae and tronchuda cabbage host

The phenolic composition of the tronchuda cabbage external leaves is well defined in a previous work, analysing several samples, from both organic and conventional agricultural practices (Ferreres et al., 2005). The composition of the host leaves, from which the larvae feed, revealed to be similar to that described before, being detected 13 kaempferol derivatives (Table 3). The flavonoid profile obtained with *P. brassicae* was then compared with that of the cabbage. Kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside-7-*O*-sophoroside and kaempferol 3-*O*-sophoroside were the only compounds that the larvae and cabbage had in common.

Although the glycosylation pattern of the flavonols is the same in both extracts, it can be observed that the flavonol 3-*O*-glycosides represent more than ca. 50% in the larvae extract (Table 4), while they correspond to ca.12% of their food plant (Table 3). This can be ascribed to the

Table 3
Phenolic composition of tronchuda cabbage external leaves

Compound	%
21+ Kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside	7.6
22 Kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside	
2 Kaempferol 3-*O*-sophoroside-7-*O*-glucoside	22.9
23 Kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside	1.4
3+ Kaempferol 3-*O*-sophoroside-7-*O*-sophoroside	11.4
24 Kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside	
25 Kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside	17.1
26 Kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside	27.8
27+ Kaempferol 3-*O*-sophorotrioside	5.1
28 Kaempferol 3-*O*-(sinapoyl)-sophoroside	
29 Kaempferol 3-*O*-(feruloyl)-sophorotrioside	0.4
30 Kaempferol 3-*O*-(feruloyl)-sophoroside	1.1
15 Kaempferol 3-*O*-sophoroside	5.2

Table 4
Phenolic composition of *P. brassicae*

Compound	%
1 Quercetin 3-*O*-sophoroside-7-*O*-glucoside	8.7
2 Kaempferol 3-*O*-sophoroside-7-*O*-glucoside	10.0
3 Kaempferol 3-*O*-sophoroside-7-*O*-sophoroside	6.6
4 Quercetin 3-*O*-(feruloyl)-triglucoside-7-*O*-glucoside	4.5
5 Kaempferol 3-*O*-(sinapoyl)-triglucoside-7-*O*-glucoside	5.0
6 Kaempferol 3-*O*-(feruloyl)-triglucoside-7-*O*-glucoside	5.6
7 Kaempferol 3-*O*-(*p*-coumaroyl)-triglucoside-7-*O*-glucoside	2.6
8 Kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside-7-*O*-glucoside	0.5
9 Kaempferol 3-*O*-(caffeoyl)-sophoroside-7-*O*-glucoside	1.8
10 Quercetin 3-*O*-(*p*-coumaroyl)-sophoroside	3.4
11 Kaempferol 3-*O*-(*p*-coumaroyl)-triglucoside	3.3
12 Kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside	13.4
13+ Kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside	9.2
14 Quercetin 3-*O*-sophoroside	
15 Kaempferol 3-*O*-sophoroside	15.8
16 Kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside (isomer)	2.4
17 Kaempferol 3-*O*-(disinapoyl)-triglucoside-7-*O*-glucoside	2.1
18 Kaempferol 3-*O*-(feruloyl/sinapoyl)-triglucoside-7-*O*-glucoside	1.3
19 Quercetin 3-*O*-(feruloyl)-triglucoside	1.9
20 Kaempferol 3-*O*-glucoside	1.9

metabolism of the flavonols glycosylated at 3 and 7 positions present on tronchuda cabbage, or to a higher efficiency of sequestration of flavonol 3-*O*-glycosides.

Among the flavonol glycoside derivatives without acylation, kaempferol 3-*O*-sophoroside is the most abundant, corresponding to ca. 16% of the total amount of phenolic compounds of the larvae (Table 4), while in tronchuda cabbage leaves it only represented ca. 5% (Table 3). This difference possibly results from the metabolism of kaempferol 3-*O*-sophoroside-7-*O*-glucoside and its acylated derivatives, which are the most abundant compounds of tronchuda cabbage external leaves.

In what concerns the presence of quercetin derivatives, the composition of the external leaves of tronchuda cabbage (Ferreres et al., 2005) was re-analysed and these were detected in vestigial amounts, which also happened with the tronchuda cabbage external leaves eaten by *P. brassicae*. The larvae contain high amounts of quercetin derivatives (ca. 18% of the total amount of phenolic compounds) (Table 4), while in tronchuda cabbage these compounds are present only in trace amounts, suggesting that *P. brassicae* selectively sequester these flavonoids or that the kaempferol glycosides are metabolised into quercetin glycosides by the larvae.

The presence of *p*-coumaroyl derivatives, which have not been found on either the internal or external leaves of tronchuda cabbage (Ferreres et al., 2005; Sousa et al., 2005) can be explained by the demethoxylation of the sinapoyl and/or feruloyl derivatives during the metabolism process in the larvae. On the other hand, the absorbance of the peaks observed in Fig. 1 for acylated flavonoid derivatives, cannot be taken as proportional to their abundance, as some of them co-eluted with other unidentified cinnamoyl acids derivatives, presenting a similar UV spectrum and contributing to the overall absorbance of those peaks. Another possible explanation is their existence in tronchuda cabbage leaves in concentrations below the detection limits, being, however, selectively uptaken and accumulated by *P. brassicae*.

The existence of two methoxylated flavonol derivatives in *P. brassicae* (compounds **8** and 1**3**) (Fig. 4) can be resultant from the metabolism of kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside present on the external leaves of the tronchuda cabbage.

As far as we know, this is the first report about the uptake of flavonoids by *P. brassicae* and it was observed they sequestered flavonoids from tronchuda cabbage external leaves. In addition, some of them may undergo metabolism during ingestion. The results suggest that *P. brassicae* may have interest for the synthesis and/or accumulation of potential health promoting compounds, which are rather unusual in nature. Although the *P. brassicae* gut was not removed and longer starving periods should be tested, some conclusions could be obtained as larvae and feeding plant contain only three compounds in common, indicating that the 17 different phenolics found in larvae are directly related with the insect, being a consequence of its metabolism and/or selective sequestration. Further studies should be done, varying the starving periods before freezing and analysing gut and remaining body separately, in order to obtain more information about the metabolic process. Larvae in different instars should also be studied, to evaluate the effect of the developmental stage in sequestration rate of flavonoids. Also the study of the faeces of larvae can provide valuable data about the excretion of flavonoids.

3. Experimental

3.1. Standards and reagents

Kaempferol 3-*O*-rutinoside, kaempferol 3-*O*-glucoside and quercetin 3-*O*-rutinoside were from Extrasynthése (Genay, France). Methanol, formic and acetic acid were purchased from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

3.2. Larvae, plant material and sampling

Wild *P. brassicae* larvae (fourth instar) and respective tronchuda cabbage external leaves (of three individuals with 45 days-old) host plants were collected on fields located in Samil, Bragança, northeastern Portugal. Voucher specimens of tronchuda cabbage leaves are deposited at Serviço de Farmacognosia from Faculdade de Farmácia, Universidade do Porto. After collection the larvae were kept without food for 1 h before they were frozen. The frozen larvae and plant material were freeze-dried and kept in a dessicator until analysis.

3.3. Extraction of the phenolic compounds

The identification of the phenolic compounds was performed using a hydromethanolic extract of the lyophilised larvae: ca. 1.5 g powdered larvae was thoroughly mixed with 2.5 ml methanol–water (1:1), ultra-sonicated and filtered.

The same extraction methodology was used for quantification purposes of phenolic compounds in larvae and plant material.

3.4. HPLC-DAD-MS/MS-ESI qualitative analysis

Chromatographic separations were carried out on a 250 mm × 4 mm, 5-μm particle size, RP-18 LiChroCART (Merck, Darmstadt, Germany) column protected with a 4 mm × 4 mm LiChroCART guard column using acetic acid 1% (A) and methanol (B) as solvents, starting with 20% B and using a gradient to obtain 50% B at 35 min. The flow rate was 1 ml min^{-1} and the injection volume was 20 μl. The HPLC system was equipped with an Agilent 1100 Series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, an G1313A autosampler, a G1322A

degasser and a G1315B photodiode array detector controlled by a ChemStation software (Agilent, v. 08.03). Spectroscopic data from all peaks were accumulated in the range 240–400 nm, and chromatograms were recorded at 330 nm. The mass detector was a G2445A Ion-Trap Mass Spectrometer equipped with an electrospray ionisation (ESI) system and controlled by LCMSD software (Agilent, v. 4.1.). Nitrogen was used as nebulising gas at a pressure of 65 psi and the flow was adjusted to 11 l min^{-1}. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full-scan mass covered the range from m/z 90 up to m/z 2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS data were acquired in the negative ionisation mode. MS^n data were achieved in the automatic mode on the more abundant fragment ion in MS^{n-1}. Tables 1 and 2 show the most frequent ions which characterise the fragmentation of the compounds. Other ions were found but they have not been included due to their low significance on the MS behaviour ions.

3.5. HPLC-DAD quantitative analysis

Twenty microliters of each extract were analysed using a HPLC unit (Gilson) and a 250 × 4.6 mm i.d., 5 μm Spherisorb ODS2 column (Waters, Milford, USA). The solvent system was a mixture of formic acid 5% in water (A) and methanol (B), with a flow rate of 1 ml min^{-1}, and the gradient was as follows: 0 min – 10% B; 25 min – 20% B; 40 min – 50% B; 45 min – 50% B; 46 min – 90% B; 50 min – 90% B; 55 min – 100% B; 58 min – 100% B; and 60 min – 10% B. Detection was achieved with a Gilson diode array detector. Spectroscopic data from all peaks were accumulated in the range of 200–400 nm, and chromatograms were recorded at 330 nm. The data were processed on Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the peak areas recorded in the chromatograms relative to that registered for known concentrations of external standards. With the exception of kaempferol 3-*O*-glucoside, which was quantified as it, the other kaempferol and quercetin derivatives were quantified as kaempferol 3-*O*-rutinoside and quercetin 3-*O*-rutinoside, respectively, since none of the identified compounds was commercially available.

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4.17. *Pieris brassicae* inhibits xanthine oxidase[1]

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Pieris brassicae Inhibits Xanthine Oxidase

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The antioxidant potential of an aqueous extract obtained from *Pieris brassicae* larvae reared on *Brassica oleracea* L. var. *costata* DC was evaluated against 2,2-diphenyl-1-picrylhydrazyl radical and several reactive oxygen species. The results revealed an effective concentration-dependent protective activity against superoxide and hydroxyl radicals, being superior to that of the host plant. In addition, the larvae extract also exhibited a strong inhibitory effect on xanthine oxidase that was not observed for *B. oleracea* var. *costata*. A weak scavenging ability was noticed for hypochlorous acid. Several phenolic compounds with complex chemical structures that are hard to synthesize in the laboratory were found in *P. brassicae* extract. This is the first time that an insect has been tested for its xanthine oxidase inhibitory capacity, which proved to be very high. These findings are interesting considering that they can be used by food or pharmaceutical industries to prevent the oxidation of their products, to increase the dietary supply of antioxidants, or for prevention of free radical-mediated diseases, namely, gout.

KEYWORDS: ***Pieris brassicae*; *Brassica oleracea* var. *costata*; phenolic compounds; antioxidant activity; xanthine oxidase**

INTRODUCTION

Oxidants relevant to human diseases come from normal intracellular biological functions, inflammatory processes, and exposure to xenobiotics, either because they have pro-oxidant activity or because they induce the formation of other oxidative agents in cell (*1*). Because of the facility of those agents to accept electrons from target molecules, they are able to modify their structure or function. Thus, oxidants can interact with the membranes, genetic material, and enzymatic processes. Additionally, they can change extracellular media, modifying tissue architectures, defense molecules, and cellular mediators. In fact, oxidants are able to change the structure and/or function of biological important molecules, like nucleic acids, lipids, proteins, and carbohydrates (*1*−*3*).

Although essential to aerobic organisms, oxygen exhibits undesirable effects due to the formation of reactive oxygen species (ROS) in every cell with aerobic metabolic activity. To maintain the homeostasis, oxidants are inactivated by an array of intra- and extracellular antioxidants. Disequilibrium in the oxidant/antioxidant status causes oxidative stress, resulting in many pathophysiologic conditions (*2, 3*).

The importance of natural antioxidants is well-established, being of great interest for health, nutritional, and food purposes. To deal with ROS, several antioxidant defenses have arisen from insects. Their role is to keep low steady-state levels of ROS and other radicals in the cell. The enzymes superoxide dismutase, catalase, glutathione reductase, selenium-dependent glutathione peroxidase, selenium-independent glutathione peroxidase, and the glutathione-*S*-transferases constitute an essential defense line against radicals (*4*−*6*).

The insects also possess low molecular weight antioxidants. The glutathione, besides its role as a substrate for glutathione reductase, glutathione peroxidase, and the glutathione-*S*-transferases, is a scavenger of hydroxyl and singlet oxygen, can reactivate some enzymes inhibited under oxidizing conditions, and is implicated in vitamin E regeneration (*5*). The role of glutathione as an antioxidant defense mechanism was assessed in several insect species, namely, in *Epiblema scudderiana* (*5*), *Mayetiola destructor* (*6*), *Melanoplus sanguinipes*, and *Aulocara ellioti* (*7*). Within phytochemicals, phenolic compounds, namely, flavonoids, are recognized for their antioxidative capacity (*8*). The presence of flavonoids in insects is positively associated with their existence in the feeding vegetal material once insects

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are unable to synthesize flavonoids or their precursors de novo (*9, 10*).

Larvae of *Pieris brassicae* L. (Lepidoptera: Pieridae) insects are specialists on crucifers, feeding on a variety of Brassicaceae species. The phenolic profiles of *P. brassicae* larvae reared on the leaves of *Brassica oleracea* L. var. *costata* DC and of *Brassica rapa* var. *rapa* L. have been determined before by high-performance liquid chromatography−diode array detection−mass spectrometry/mass spectrometry−electrospray ionization (HPLC-DAD-MS/MS-ESI) (*11, 12*). Several flavonoids, with complex structures that are hard to synthesize, were found in the larvae extracts. In addition to the determinant role displayed by the feeding material composition, those studies made it possible to determine that the larvae has the ability to sequester and metabolize the phenolic compounds present in the two host plants.

Previous works concerning *B. oleracea* var. *costata* revealed the antioxidant capacity of this species, for which its phenolic compounds contributed (*13−15*). Despite the occurrence of a high content of flavonoids not detected in *B. oleracea* var. *costata*, the antioxidant potential of *P. brassicae* reared on this matrix has not so far been assessed. Only the antiradical capacity of *P. brassicae* fed with *B. rapa* var. *rapa* (with 12 h food privation) against 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide radical, and nitric oxide was evaluated in chemical systems (*16*).

The aim of the work herein was to achieve further knowledge on the antioxidant capacity of *P. brassicae*, a frequent pest of some *Brassica* species. For this purpose, aqueous extracts of the larvae with 1 h food privation and of *B. oleracea* var. *costata* host plant were studied for their capacity to act as scavengers of several ROS (superoxide radical, hydroxyl radical, and hypochlorous acid), chemically and enzymatically generated.

MATERIALS AND METHODS

Reagents. Kaempferol 3-*O*-rutinoside was from Extrasynthése (Genay, France). Methanol and formic were purchased from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA). DPPH, xanthine (X), xanthine oxidase (XO) grade I from buttermilk (EC 1.1.3.22), β-nicotinamide adenine dinucleotide reduced form (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), anhydrous ferric chloride ($FeCl_3$), ethylenediaminetetraacetic acid disodium salt (EDTA), ascorbic acid, trichloroacetic acid, thiobarbituric acid, deoxyribose, sodium hypochlorite solution with 4% available chlorine (NaOCl), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), glutathione (GSH), glutathione disulfide (GSSG), glutathione reductase (EC 1.6.4.2), and L-cysteine were obtained from Sigma Chemical Co. (St. Louis, MO).

Larvae and Host Plant Material. Wild *P. brassicae* larvae at the fourth instar and respective *B. oleracea* var. *costata* host plant external leaves (with 45 days old) were collected in Bragança (northeastern Portugal). Larvae and plant material were immediately transported to the laboratory. *B. oleracea* var. *costata* leaves were frozen at −20 °C. The larvae were deprived from food for 1 h and then frozen. The frozen larvae and plant material were then freeze-dried.

Extracts Preparation. For the antioxidant capacity screening, aqueous extracts were prepared as follows: 0.4 g of dried larvae and 2 g of dried *B. oleracea* var. *costata* leaves were boiled for 30 min in 400 mL of water and filtered over a Büchner funnel. The resulting extracts were then frozen and lyophilized. The lyophilized extracts were kept in an exsicator, in the dark.

HPLC-DAD Analysis. The determination of the phenolic compounds in the aqueous lyophilized extracts was performed as previously reported (*11*). Briefly, 20 μL of each lyophilized extract redissolved in water was analyzed in a HPLC unit (Gilson), using a Spherisorb ODS2 (250 mm × 4.6 mm, 5 μm particle size) and a flow rate of 1 mL/min. The solvent system was a mixture of formic acid 5% (A) and methanol (B), and the gradient was as follows: 0 min, 10% B; 25 min, 20% B; 40 min, 50% B; 45 min, 50% B; 46 min, 90% B; 50 min, 90% B; 55 min, 100% B; 58 min, 100% B; and 60 min, 10% B. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200−400 nm, and chromatograms were recorded at 330 nm. Phenolic compounds were identified by comparing their UV spectra and retention times with data previously reported (*11*). The data were processed on a Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities. Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. The compounds were quantified as kaempferol 3-*O*-rutinoside.

DPPH Scavenging Activity. The disappearance of $DPPH^{\bullet}$ was monitored spectrophotometrically at 515 nm on a Multiskan Ascent plate reader (Thermo Electron Corp.), following a described procedure (*15*). For each extract, a dilution series was prepared in a 96 well plate. The reaction mixtures in the sample wells consisted of 25 μL of lyophilized extract and 200 μL of 150 μM DPPH dissolved in methanol. The plate was incubated for 30 min at room temperature after addition of DPPH. Three experiments were performed in triplicate.

Superoxide Radical Scavenging Activity. The effect of the lyophilized extracts on the superoxide radical-induced reduction of NBT was monitored spectrophotometrically in a Multiskan Ascent plate reader (Thermo Electron Corp.), in kinetic function, at 562 nm.

Nonenzymatic Assay. Superoxide radicals were generated by the NADH/PMS system, as previously reported (*15*). All components were dissolved in phosphate buffer (19 mM, pH 7.4). For each extract, five different concentrations were tested. Three experiments were performed in triplicate.

Enzymatic Assay. Superoxide radicals were generated by the X/XO system following a described procedure (*15*). Briefly, X was dissolved in NaOH (1 μM) and subsequently in phosphate buffer (50 mM) with EDTA (0.1 mM, pH 7.8), XO in EDTA (0.1 mM), and the other components in phosphate buffer (50 mM) with EDTA (0.1 mM, pH 7.8). For each extract, a dilution series was assayed. Three experiments were performed in triplicate.

Effect on XO. The effect of the lyophilized extracts on XO activity was evaluated by measuring the formation of uric acid from X in a double beam spectrophotometer (Heλios α, Unicam), at room temperature, as before (*15*). The reaction mixtures contained the same components of the enzymatic assay at the same proportion, except for NBT. The absorbance was measured at 295 nm for 2 min. Three experiments were performed in triplicate.

Effect of GSH. To check for the possibility of GSH from larvae extract or fragments resulting from its degradation to interfere with XO, an aqueous solution of GSH at the concentration corresponding to the IC_{25} value found in the evaluation of the effect of lyophilized larvae extract on the enzyme was prepared and submitted to boiling for 30 min, as it happened for the larvae.

The effect of boiled GSH solution against superoxide radical generated by the enxymatic system and on XO activity was evaluated as indicated above for larvae lyophilized extract. The GSH content in boiled GSH solution was determined by the DTNB-GSSG reductase recycling assay, as described before (*17, 18*), at 415 nm.

Hydroxyl Radical Scavenging Activity. The deoxyribose method for determining the scavenging effect of the aqueous extracts on hydroxyl radicals was performed according to a described procedure (*15*) in a double beam spectrophotometer (Heλios α, Unicam). Reaction mixtures contained ascorbic acid, $FeCl_3$, EDTA, H_2O_2, deoxyribose, and lyophilized extracts. All components were dissolved in KH_2PO_4−KOH buffer (10 mM, pH 7.4). This assay was also performed without either ascorbic acid or EDTA, to evaluate the extracts prooxidant and metal chelation potential, respectively. For each extract, five different concentrations were tested. Three experiments were performed in triplicate.

Hypochlorous Acid Scavenging Activity. The inhibition of hypochlorous acid-induced TNB oxidation to DTNB was evaluated as previously reported (*15*), in a double beam spectrophotometer (Heλios

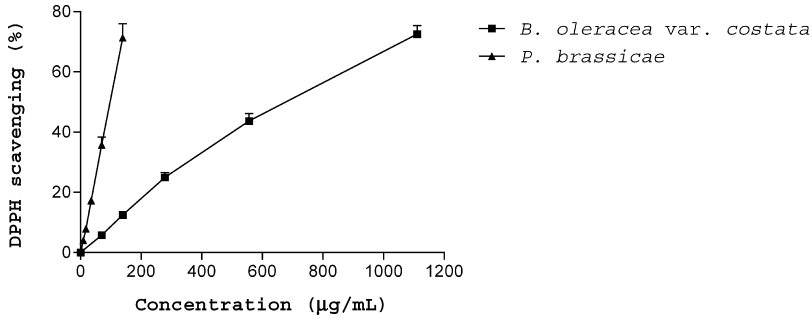


Figure 1. Effect of *P. brassicae* and *B. oleracea* var. *costata* on DPPH radical reduction. Values show means ± SEs from three experiments performed in triplicate.

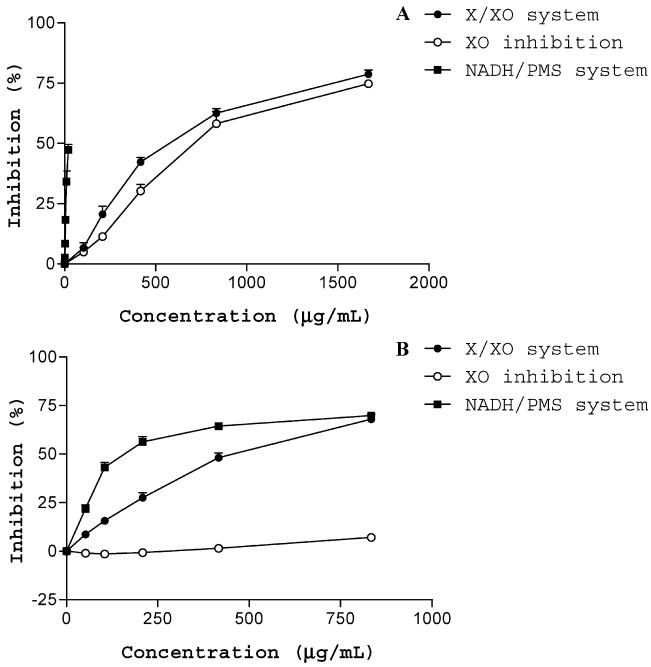


Figure 2. Effect of (**A**) *P. brassicae* and (**B**) *B. oleracea* var. *costata* against superoxide radical and on XO. Values show means ± SEs from three experiments performed in triplicate.

α, Unicam). Hypochlorous acid and TNB were prepared immediately before use. For each extract, a dilution series was prepared. Three experiments were performed in triplicate.

RESULTS AND DISCUSSION

DPPH is a stable free radical, able to accept one electron or hydrogen atom, turning it into a nonradical species, hardly oxidizable. The DPPH assay provides basic information on the antiradical activity of extracts (*19*). A microassay was performed in which the scavenging of DPPH was followed by monitoring the decrease in absorbance at 515 nm, which occurs due to the reduction by the antioxidant. The *P. brassicae* aqueous extract exhibited a strong antioxidant activity, in a concentration-dependent way (IC_{50} at 97 μg/mL), being much more effective than the host plant (IC_{50} at 678 μg/mL) (**Figure 1**).

Superoxide radical is the first product of oxygen univalent reduction. Activated phagocyte cells generate this ROS, which is important to allow killing of some of the bacterial strains that they engulf. Some superoxide radical is accidentally formed in vivo: Some of the electrons passing through the respiratory chain leak from the electron carriers and pass directly onto oxygen, reducing it (*20*). XO is also involved in the in vivo production of superoxide radical, by catalyzing the conversion of hypoxanthine in X and of X into uric acid (*21*). The biological

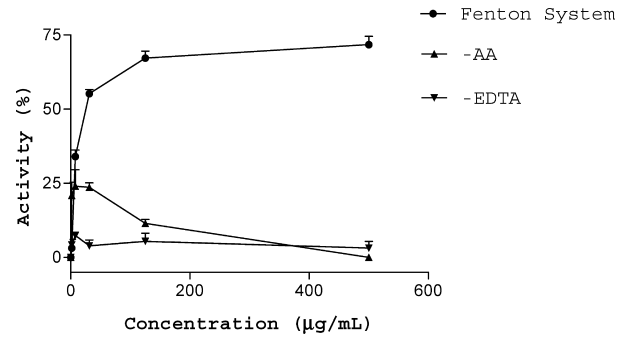


Figure 3. Effect of *P. brassicae* against hydroxyl radical, pro-oxidant activity (−AA), and metal chelating capacity (−EDTA). Values show means ± SEs from three experiments performed in triplicate.

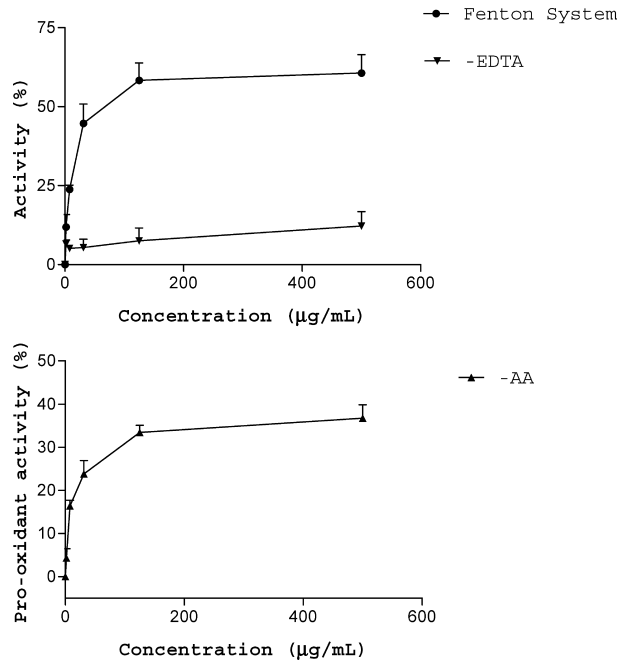


Figure 4. Effect of *B. oleracea* var. *costata* against hydroxyl radical, pro-oxidant activity (−AA), and metal chelating capacity (−EDTA). Values show means ± SEs from three experiments performed in triplicate.

significance of superoxide radical is mainly related with its conversion into much more reactive species, like hydroxyl radical and peroxynitrite (*22*).

Superoxide radical was generated by the enzymatic X/XO and by the chemical phenazine methosulphate/NADH (PMS/NADH) systems. The prevention of NBT reduction to the chromogen formazan, induced by superoxide radical, was used as the measured end point. Taking into account that an inhibitory effect on the enzyme itself would also lead to a decrease in

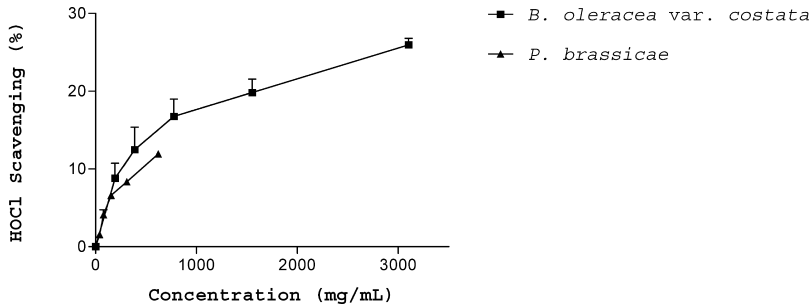


Figure 5. Effect of *P. brassicae* and *B. oleracea* var. *costata* against hypochlorous acid. Values show means ± SEs from three experiments performed in triplicate.

NBT reduction (*23*), the effect of the extracts on the metabolic conversion of X to uric acid was also studied.

An effective concentration-dependent antioxidant capacity was found for both *P. brassicae* and *B. oleracea* var. *costata* leaf lyophilized extracts, which exhibited superoxide radical scavenging activity using the X/XO system, with an IC_{25} at 251 and 186 μg/mL, respectively (**Figure 2**). However, the larvae displayed a potent inhibitory effect on XO, in a concentration-dependent manner (IC_{25} at 358 μg/mL), while *B. oleracea* var. *costata* had no effect on this enzyme (**Figure 2**). It is the first time that an insect is reported to possess XO inhibitory capacity. These findings can be of great relevance in the case of gout disease, in which high levels of uric acid are involved (*21*). As it was not possible to show a clear-cut scavenging effect on superoxide radical for the larvae, the effect of both extracts on superoxide radical generated in PMS/NADH system was determined. A concentration-dependent scavenging effect was observed, which was noticeably superior for the larvae extract: IC_{25} at 7.4 and 59 μg/mL, for larvae and *B. oleracea* var. *costata*, respectively (**Figure 2**). According to these results, it may be anticipated that *P. brassicae* exerts an effective protective role against superoxide radical by acting as both scavenger and XO inhibitor.

Within free radicals and other ROS, hydroxyl radical is the most unstable and reactive: It shows a great oxidative power and rapidly combines with almost all molecules in its immediate vicinity. The formation of hydroxyl radical requires traces of transition metal ions, among which iron and copper seem likely to be the most important in vivo (*20, 22*). Exposure to ionizing radiation, peroxynitrite protonation, and decomposition and the reaction of hypochlorous acids with superoxide radical are also sources of hydroxyl radical (*22*).

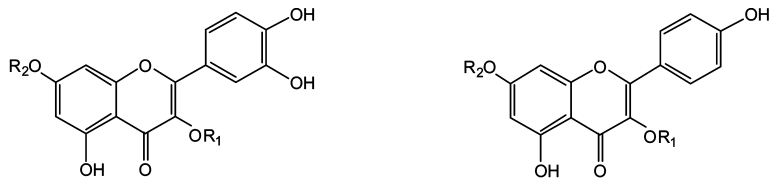
Hydroxyl radical was generated by the Fe^{3+}-EDTA/ascorbate Fenton system and assayed by evaluating deoxyribose degradation into thiobarbituric acid-reactive substances. The lyophilized extract of *P. brassicae* revealed scavenging activity for hydroxyl radical, in a concentration-dependent way (IC_{25} at 6.1 μg/mL) (**Figure 3**), being superior to that exhibited by *B. oleracea* var. *costata* (IC_{25} at 9.2 μg/mL) (**Figure 4**). If we omit ascorbate in the reaction system and if pro-oxidant compounds exist, they will promote the formation of hydroxyl radical by redox cycling the metal ion required for its generation, causing deoxyribose degradation (*24*). In the assay performed under these conditions, some pro-oxidant effect was observed for concentrations of larvae extract higher than 7.8 μg/mL (**Figure 3**), while the cabbage was revealed to have pro-oxidant activity at all tested concentrations (**Figure 4**). Inhibition of iron-dependent deoxyribose degradation in the absence of EDTA depends not only on the ability of a scavenger to react with hydroxyl radicals but also on its capacity to form complexes with iron ions (*25*). Under these circumstances, either *P. brassicae* or *B. oleracea* var. *costata* extracts showed no metal chelating activity (**Figures 3** and **4**).

Hypochlorous acid is produced by the oxidation of Cl^- ions catalyzed by neutrophil-derived myeloperoxidase, in the presence of hydrogen peroxide. It damages and induces target cell lysis, caused by sulfydril oxidation, in plasma membrane proteins, inactivates α_1-antiprotease and antioxidants enzymes like catalase, and activates collagenase and gelatinase (*20, 26−28*).

The hypochlorous acid scavenging activity was tested by measuring the inhibition of hypochlorous acid-induced 5-thio-2-nitrobenzoic acid (TNB) oxidation to DTNB. *P. brassicae* aqueous extract showed some concentration-dependent protective activity (IC_{10} at 453 μg/mL), although it was less effective than *B. oleracea* var. *costata* (IC_{10} at 257 μg/mL) (**Figure 5**).

In a general way, and according to the results obtained in all assays, *P. brassicae* was revealed to be a more effective antioxidant than its host plant. The phenolics profile of the two analyzed extracts was identical to that described before (*11*), being composed by flavonol derivatives glycosilated at 3 or simultaneously at 3 and 7 positions, with some of them acylated (**Figures 6−8**). Kaempferol 3-*O*-sophoroside was the main compound in *P. brassicae* aqueous lyophilized extract, while kaempferol 3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside was the main compound in that of the host *B. oleracea* var. *costata* (**Tables 1** and **2**). Although the last presented higher phenolics content, the distinct qualitative composition seems to be determinant for the antioxidant potential exhibited by the two analyzed extracts. In fact, either *P. brassicae* or *B. oleracea* var. *costata* extracts contained several flavonol derivatives, but only kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside-7-*O*-sophoroside, and kaempferol 3-*O*-sophoroside are common to both.

Flavonoids have been identified as fulfilling most of the criteria involved in the mechanisms of antioxidant action, which include suppressing ROS formation, either by inhibition of enzymes or chelation of trace elements involved in free radical production, scavenging ROS, and upregulating or protecting antioxidant defenses (*29*). Flavonol glycosides have already been demonstrated to possess antioxidant capacity (*30, 31*). As observed in the previous work (*11*), despite the identical glycosylation pattern, the larva exhibited a higher relative amount of 3-*O*-glycosides (994 mg/kg as compared to 497 mg/kg found in tronchuda cabbage extract), probably by hydrolysis by the larva. This seems to suggest that flavonol 3-*O*-glucosides contribute to a greater extent to the strongest protective effects displayed by *P. brassicae*. In fact, it is known that the addition of a second glycoside residue decreases the activity due to steric hindrance by addition of sugar moieties (*19*). Additionally, the presence of higher amounts of quercetin derivatives in *P. brassicae* extract (around 360 mg/kg) can also, at least partially, explain its highest antioxidative properties once it is well-established that quercetin is a more potent antioxidant than kaempferol, due to the presence of a cathecol group in the B ring of the former (*29*). Indeed, as before (*11*), only trace



1 R_1 = sophoroside; R_2 = glucoside
4 R_1 = (feruloyl)triglucoside; R_2 = glucoside
10 R_1 = (*p*-coumaroyl)sophoroside; R_2 = H
14 R_1 = sophoroside; R_2 = H
19 R_1 = (feruloyl)triglucoside; R_2 = H

2 R_1 = sophoroside; R_2 = glucoside
3 R_1 = sophoroside; R_2 = sophoroside
5 R_1 = (sinapoyl)triglucoside; R_2 = glucoside
6 R_1 = (feruloyl)triglucoside; R_2 = glucoside
7 R_1 = (*p*-coumaroyl)triglucoside; R_2 = glucoside
8 R_1 = (methoxycaffeoyl)sophoroside; R_2 = glucoside
9 R_1 = (caffeoyl)sophoroside; R_2 = glucoside
11 R_1 = (*p*-coumaroyl)triglucoside; R_2 = H
12, 16 R_1 = (*p*-coumaroyl)sophoroside; R_2 = H
13 R_1 = (methoxycaffeoyl)sophoroside; R_2 = H
15 R_1 = sophoroside; R_2 = H
17 R_1 = (disinapoyl)triglucoside; R_2 = glucoside
18 R_1 = (feruloyl/sinapoyl)triglucoside; R_2 = glucoside
20 R_1 = glucoside; R_2 = H
21 R_1 = sophorotrioside; R_2 = glucoside
22 R_1 = (methoxycaffeoyl/caffeoyl)sophoroside; R_2 = glucoside
23 R_1 = sophorotrioside; R_2 = sophoroside
24 R_1 = tetraglucoside; R_2 = sophoroside
25 R_1 = (sinapoyl/caffeoyl)sophoroside; R_2 = glucoside
26 R_1 = (feruloyl/caffeoyl)sophoroside; R_2 = glucoside
27 R_1 = sophorotrioside; R_2 = H
28 R_1 = (sinapoyl)sophoroside; R_2 = H
29 R_1 = (feruloyl)sophorotrioside; R_2 = H
30 R_1 = (feruloyl)sophoroside; R2 = H

Figure 6. Structures of the phenolic compounds identified in *P. brassicae* and *B. oleracea* var. *costata*. The identities of compounds are as in **Tables 1** and **2**.

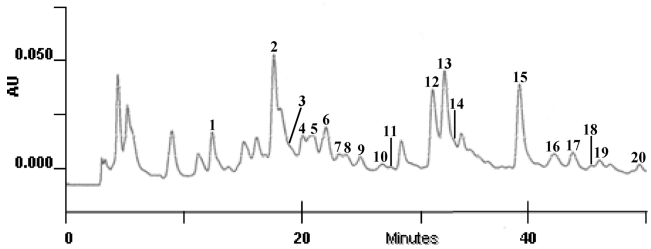


Figure 7. HPLC-DAD phenolic profile of larvae extract. Detection at 330 nm. The identities of compounds are as in **Table 1**.

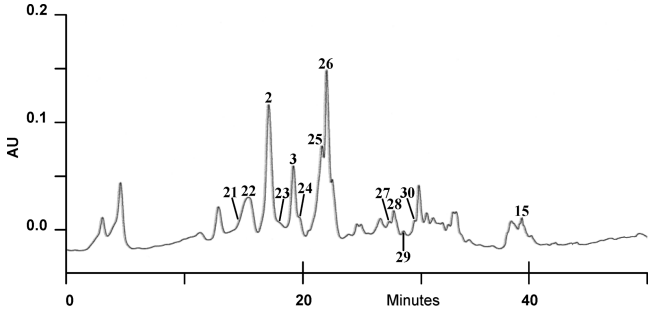


Figure 8. HPLC-DAD phenolic profile of tronchuda cabbage extract. Detection at 330 nm. The identities of compounds are as in **Table 2**.

Table 1. Phenolic Composition of *P. brassicae* Aqueous Lyophilized Extract

	compound	mg/kg
1	quercetin 3-*O*-sophoroside-7-*O*-glucoside	169
2	kaempferol 3-*O*-sophoroside-7-*O*-glucoside	195
3	kaempferol 3-*O*-sophoroside-7-*O*-sophoroside	128
4	quercetin 3-*O*-(feruloyl)triglucoside-7-*O*-glucoside	87
5	kaempferol 3-*O*-(sinapoyl)triglucoside-7-*O*-glucoside	97
6	kaempferol 3-*O*-(feruloyl)triglucoside-7-*O*-glucoside	108
7	kaempferol 3-*O*-(*p*-coumaroyl)triglucoside-7-*O*-glucoside	51
8	kaempferol 3-*O*-(methoxycaffeoyl)sophoroside-7-*O*-glucoside	10
9	kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside	36
10	quercetin 3-*O*-(*p*-coumaroyl)sophoroside	67
11	kaempferol 3-*O*-(*p*-coumaroyl)triglucoside	62
12	kaempferol 3-*O*-(*p*-coumaroyl)sophoroside	261
13	kaempferol 3-*O*-(methoxycaffeoyl)sophoroside +	179
14	quercetin 3-*O*-sophoroside	
15	kaempferol 3-*O*-sophoroside	307
16	kaempferol 3-*O*-(*p*-coumaroyl)sophoroside (isomer)	46
17	kaempferol 3-*O*-(disinapoyl)triglucoside-7-*O*-glucoside	41
18	kaempferol 3-*O*-(feruloyl/sinapoyl)triglucoside-7-*O*-glucoside	26
19	quercetin 3-*O*-(feruloyl)triglucoside	36
20	kaempferol 3-*O*-glucoside	36

amounts of quercetin glycosides were found in the host plant, suggesting that *P. brassicae* selectively sequesters these flavonoids or that the kaempferol derivatives are metabolized into quercetin glycosides by the larvae.

The presence of antioxidant enzymes in *Pieris* larvae extracts that may contribute to the antioxidative properties can be discarded, once they are denaturated by boiling at 100 °C for 30 min, turning them inactive. Concerning GSH, we verified that a boiled solution (358 μg/mL) was not able to inhibit uric acid formation by XO (0.45 ± 1.06% XO inhibition) but scavenged superoxide radical (51.70 ± 5.67% superoxide radical scavenging ability). Thus, it seems that the GSH protective effect against superoxide radical is related to its scavenging ability rather than its XO inhibitory capacity. In this solution, only ca. one-third of the initial total GSH was found, from which ca. 6% was in the oxidized form. This can be attributed to its alteration during boiling and also to its oxidation, since during this process it was not protected from light or oxygen exposure.

Although some GSH remained in the boiled solution, these data allow us to discard the possibility of the XO inhibition

Table 2. Phenolic Composition of *B. oleracea* var. *costata* Aqueous Lyophilized Extract

	compound	mg/kg
21	kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside +	316
22	kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside	
2	kaempferol 3-*O*-sophoroside-7-*O*-glucoside	955
23	kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside	58
3	kaempferol 3-*O*-sophoroside-7-*O*-sophoroside +	477
24	kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside	
25	kaempferol 3-*O*-(sinapoyl/caffeoyl)–sophoroside-7-*O*-glucoside	711
26	kaempferol 3-*O*-(feruloyl/caffeoyl)–sophoroside-7-*O*-glucoside	1158
27	kaempferol 3-*O*-sophorotrioside +	214
28	kaempferol 3-*O*-(sinapoyl)sophoroside	
29	kaempferol 3-*O*-(feruloyl)sophorotrioside	18
30	kaempferol 3-*O*-(feruloyl)sophoroside	48
15	kaempferol 3-*O*-sophoroside	217

observed with the larvae extract to be related with GSH: Besides thermal degradation of the tripeptide, the oxidized GSH that can result from light or oxygen exposure cannot be recycled to the reduced form by glutathione reductase, due to the temperature used, which destroys the enzyme. Regeneration of glutathione reduced form can be achieved in vivo by glutathione reductase and NADPH (*32*), which is not present in our in vitro assay.

In conclusion, *P. brassicae* larvae aqueous extract provides powerful natural antioxidants, with complex chemical structures, impossible to be synthesized in the laboratory. This study provided evidence for the first time that the insect is able to inhibit XO and to prevent hydroxyl radical and hypochlorous acid-induced damage. Because of the potent activity exhibited, the food industry may employ it to prevent the oxidation of its products, maintaining their quality and safety and extending their shelf life, or to improve their nutritional value, by incorporating the extract in foodstuffs, thus increasing the dietary supply of antioxidants. It may also be used by the pharmaceutical industry in antioxidative formulations for prevention of free radical-mediated diseases, namely, gout, or even as a preservative of other oxidizable formulations. The same can be applied to the cosmetic industry, for which it can be further used in antiaging formulations. In addition, it may constitute an economical advantage for *B. oleracea* var. *costata* producers who have great losses caused by *P. brassicae* infestations.

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PARTE III

DISCUSSÃO INTEGRADA

CONCLUSÕES

5. DISCUSSÃO INTEGRADA

As dietas ricas em produtos vegetais promovem a saúde e atenuam ou atrasam várias doenças, incluindo doenças cardiovasculares, diabetes, cancro e doenças relacionadas com o envelhecimento (201).

Os vegetais são uma fonte de nutrientes essenciais, mas também fornecem outros compostos bioactivos como os metabolitos secundários, aos quais poderão ser atribuídos muitos dos efeitos benéficos. As couves são o maior fornecedor de glucosinolatos na dieta, o que as distingue de outros vegetais (29). Além deste grupo de compostos, as couves são caracterizadas por terem quantidades elevadas de compostos fenólicos (103). Do ponto de vista nutricional, uma refeição contendo 100 g de couve tronchuda cozida contém apenas 21 kcal, tem pouca gordura (0,4 g), fornece fibra e tem 64 % do valor recomendado de vitamina C, devendo por isso ser incluída numa dieta saudável. Em Portugal, o consumo *per capita* de couves é de 20 kg / ano (dados de 2004), sendo que os portugueses consumiram 107,5 kg / ano de produtos hortícolas no mesmo período (INE, FAO).

Dado o número de metabolitos presentes no metaboloma de uma planta e a diversidade de efeitos biológicos prováveis, devem ser feitas diversas abordagens experimentais para avaliar os efeitos das classes de compostos mais importantes.

Os compostos polifenólicos estão a ganhar aceitação como sendo os principais responsáveis pelos efeitos benéficos proporcionados pelos frutos e vegetais. Apesar de ter havido progressos significativos, ainda existem muitas áreas que precisam de ser esclarecidas para chegar a conclusões definitivas sobre os mecanismos que ligam o consumo de polifenóis de origem vegetal à diminuição dos danos oxidativos e promoção da saúde (201). Os polifenóis podem agir como antioxidantes, inibidores e indutores de enzimas, inibidores das actividades dos receptores, e indutores e inibidores da expressão dos genes, entre outras acções. Ao contrário dos nutrientes que geralmente têm funções muito específicas, a acção de metabolitos secundários como os polifenóis é menos bem definida, possuindo muitas vezes funções que se sobrepõem.

A obtenção de vegetais com maior qualidade nutricional e medicinal deve ser um dos objectivos dos programas agrícolas (202). A influência de factores agronómicos no perfil metabólico das plantas deve ser avaliada e devem ser seleccionadas as condições que conduzam à obtenção de plantas potencialmente mais benéficas para a saúde. Esta preocupação com a qualidade alimentar levou, nos últimos anos, ao aumento da procura de produtos biológicos (203).

As plantas obtidas por micropropagação podem produzir e acumular muitos metabolitos secundários com interesse medicinal (204). Neste sistema de produção é possível ter um maior controlo das vias biossintéticas e aumentar a produção e acumulação de compostos antioxidantes, por exemplo optimizando a composição do meio de cultura e utilizando fitoreguladores. Também é possível obter compostos novos na natureza ou que não são produzidos pela planta em estado selvagem (204).

Infelizmente, os efeitos potencialmente tóxicos do consumo excessivo de compostos como os flavonóides são muitas vezes ignorados. Em doses elevadas, os flavonóides podem comportar-se como mutagénicos, pró-oxidantes e inibidores de enzimas chave envolvidas no metabolismo das hormonas. Assim, em doses elevadas, os efeitos adversos dos flavonóides podem sobrepor-se aos efeitos benéficos, sendo necessário ter algum cuidado quando se pensa em ingerir doses superiores às obtidas através de uma dieta típica (184). Os efeitos observados também são afectados pela variabilidade genética, interacção com outros compostos e características clínicas dos indivíduos.

Neste capítulo serão discutidos os resultados obtidos na caracterização do perfil metabólico da couve tronchuda e a sua relação com o potencial antioxidante desta matriz.

5.1. Compostos fenólicos

A biossíntese e variedade de compostos fenólicos nas plantas são influenciadas por numerosos factores incluindo a luz, factores genéticos, condições ambientais, germinação, grau de maturação, processamento e armazenamento (159). Estes factores influenciam a qualidade nutricional dos alimentos e os possíveis efeitos benéficos nos consumidores (70).

Os trabalhos realizados no âmbito desta tese de doutoramento permitiram caracterizar o perfil de compostos fenólicos da couve tronchuda e avaliar alguns factores que influenciam este perfil tais como a parte da planta estudada, o tipo de cultivo, a época de colheita e o tipo de extracção. O perfil fenólico de couve tronchuda obtida por micropropagação com diferentes meios e fitoreguladores também foi caracterizado.

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por micropropagação com diferentes meios e fitoreguladores também foi caracterizado.

Durante o cultivo algumas plantas foram atacadas por borboletas da espécie *P. brassicae*, cuja larva se alimenta exclusivamente das folhas de couve onde os ovos são depositados. Tirando vantagem desta aparente contrariedade, avaliou-se o potencial das larvas sequestrarem e metabolizarem os compostos fenólicos presentes no seu alimento. A couve que serviu de alimento à larva também foi caracterizada para verificar alguma resposta no perfil fenólico ao ataque da larva (metabonómica).

5.1.1. Caracterização do perfil de compostos polifenólicos

A couve tronchuda caracteriza-se pela presença de quantidades elevadas de diferentes ácidos hidroxicinâmicos e flavonóides. Na base da grande variedade estão maioritariamente dois compostos comuns, o ácido sinápico e o campferol, que apresentam padrões de substituição característicos para cada uma das matrizes estudadas: sementes, [4.1, (143)]; plântulas (forma germinativa das sementes), [4.2, (205)]; folhas internas, [4.3, (135)]; folhas externas, [4.5, (11)]; inflorescências [4.12, (146)]; rebentos caulinares [4.14, (145)] e larva de *P. brassicae* [4.16, (144)]. Nestas matrizes identificaram-se 51 heterósidos flavonólicos dos quais 43 são derivados do campferol.

Na Figura 37, estão esquematizados os padrões de substituição dos ácidos hidroxicinâmicos e flavonóis para cada uma das matrizes atrás referidas. Comparando o perfil de compostos fenólicos da couve tronchuda com o de outras variedades de *B. oleracea* (Tabela 5) verifica-se que existem muitas semelhanças: todas as variedades têm um perfil fenólico muito complexo, com predominância de derivados de campferol com elevado grau de glicosilação. Mesmo assim, na caracterização do perfil fenólico da folha externa de couve tronchuda foram identificados, pela primeira vez na natureza, vários derivados do campferol (campferol 3-*O*-(metoxicafeoil/cafeoil)-soforósido-7-*O*-glucósido, campferol 3-*O*-(sinapoil/cafeoil)-soforósido-7-*O*-glucósido, campferol 3-*O*- (feruloil/cafeoil)-soforósido-7-*O*-glucósido, campferol 3-*O*-(feruloil)-soforotriósido, campferol 3-*O*-(feruloil)-soforósido e o campferol 3-*O*-tetraglucósido-7-*O*-soforósido). O elevado grau de glicosilação deste último composto (contém 6 resíduos de açúcar) é muito invulgar na natureza.

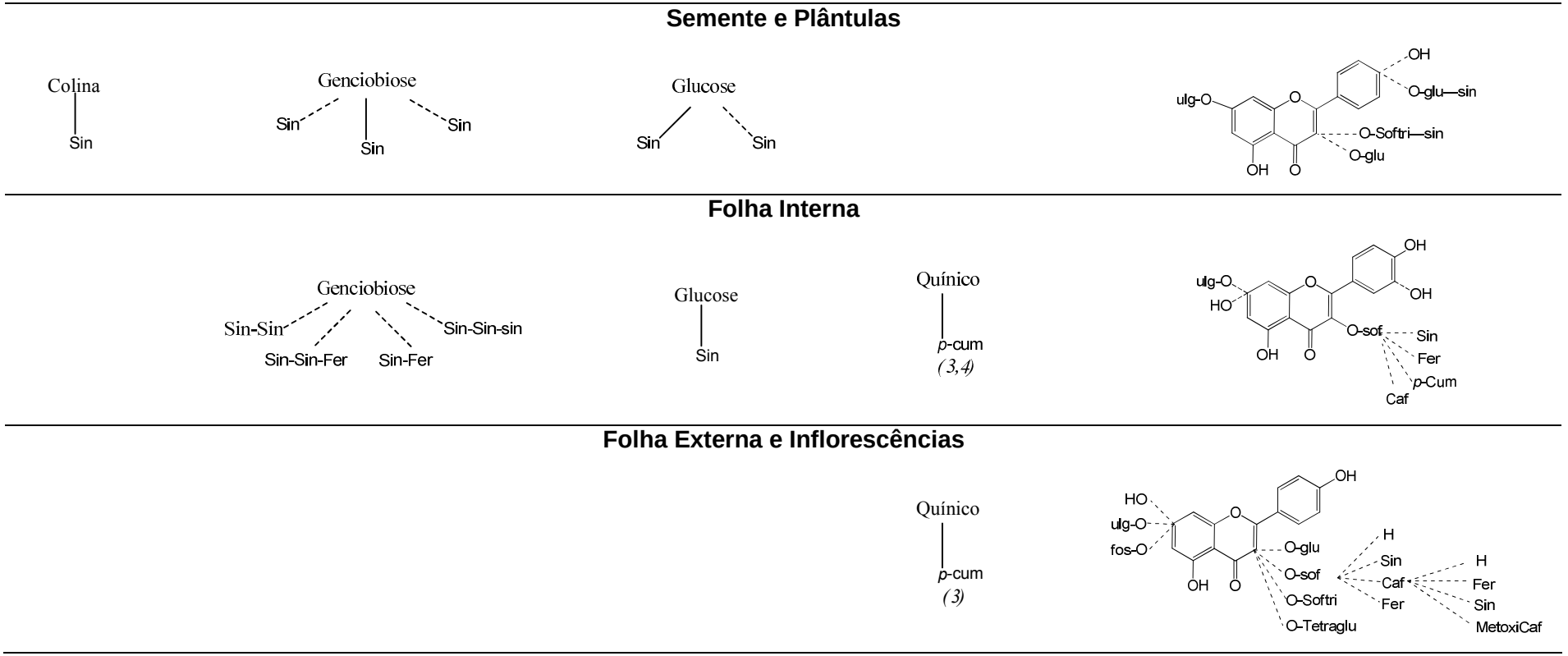

Figura 37. Padrão de substituição dos compostos polifenólicos em extractos de couve tronchuda e de larva de *P. brassicae*.

Caf: Cafeico, Fer: Ferúlico, Glu: Glucose, Metoxicaf: Metoxicafeico, *p*-cum: *p*-cumárico, Sin: Sinápico, Sof: Soforose, Softri: Soforotriose, Tetraglu: Tetraglucose. As ligações a tracejado são substituições que só se verificam nalguns compostos.

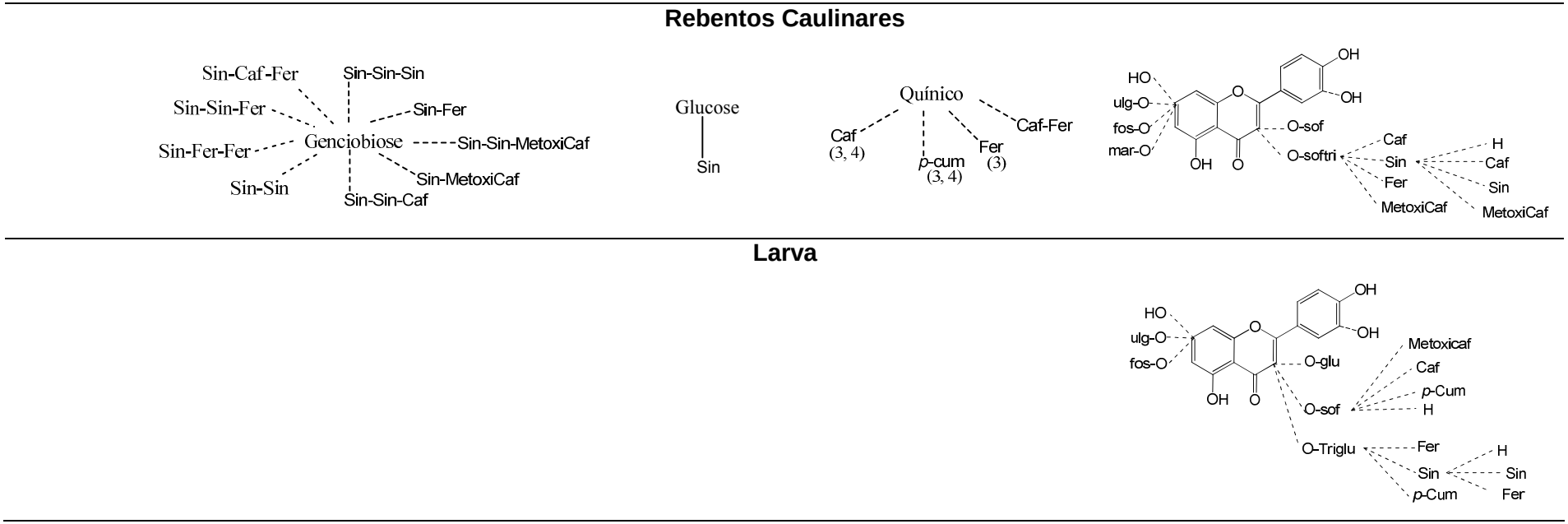

Figura 37. Padrão de substituição dos compostos polifenólicos em extractos de couve tronchuda e de larva de *P. brassicae* (cont.).

Caf: Cafeico, Fer: Ferúlico, Glu: Glucose, Metoxicaf: Metoxicafeico, *p*-cum: *p*-cumárico, Ram: Ramnose, Sin: Sinápico, Sof: Soforose, Softri: Soforotriose. As ligações a tracejado são substituições que só se verificam nalguns compostos.

5.1.1.1. Flavonóis

Como se pode observar pela Figura 37, a estrutura básica que caracteriza o perfil fenólico dos extractos aquosos de couve tronchuda é um campferol substituído nos hidroxilos dos carbonos 3 e 7 (em alguns compostos só em 3) com grupos glucosilo com um número variável de glucose, em que a cadeia glicosídica no carbono 3 em alguns dos compostos é acilado com um ou dois ácidos hidroxicinâmicos. Neste extractos também se identificaram vários heterósidos não flavonoídicos de ácidos hidroxicinâmicos e de ácidos clorogénicos.

Relativamente aos flavonóis, nas **sementes** apenas se identificaram 2 heterósidos flavonólicos, um dissubstituído e outro trisubstituído. Este último deixou de ser detectado nas plântulas logo após a germinação das sementes. Na **folha interna** foram identificados 7 compostos dos quais 6 são dissubstituídos. O número de heterósidos flavonólicos identificados aumentou para 14 na **folha externa** e nas **inflorescências**, sendo 8 deles dissubstituídos. Nos **rebentos caulinares** foram identificados 20 compostos dos quais 13 são dissubstituídos. Na **larva**, foram identificados 20 compostos sendo 11 dissubstituídos. As geninas (campferol e quercetina) não foram detectadas nas matrizes analisadas.

Relativamente à fracção glicosídica dos flavonóis, a glucose é o único açúcar presente, com excepção dos rebentos caulinares em que foram identificados dois heterósidos de campferol contendo ramnose. Isto pode indicar que a ramnose existe em maior quantidade nas culturas *in vitro*, e por isso as GT's utilizam este substracto, ou uma alteração no tipo de GT's, sendo activadas isoformas que utilizam preferencialmente a ramnose como substracto.

Os açúcares encontram-se ligados a hidroxilos (*O*-heterósidos) do carbono 3, nos compostos monosubstituídos, ou dos carbonos 3 e 7, nos compostos dissubstituídos. Apenas nas sementes foi identificado um campferol com substituição nos carbonos em 3, 7 e 4'. O padrão de substituição predominante é a dissubstituição nos hidroxilos dos carbonos 3 e 7.

Quanto ao número de açúcares, foram encontrados grupos substituintes contendo entre 1 e 4 açúcares, sendo que os tetraglucósidos apenas foram identificados em duas matrizes (folhas externas e inflorescências) e em quantidades vestigiais. O número de resíduos de açúcar dos grupos substituintes no carbono 3 é geralmente maior do que no carbono 7, sendo que em alguns compostos é igual. Não existe nenhum composto que seja substituído apenas no carbono 7.

Quando existe mais do que um resíduo de glucose, a ligação interglucosídica mais comum é 1 → 2 (soforoses e soforotrioses). Nos extractos de larva de *P. brassicae* não foi possível determinar a ligação interglucosídica em vários compostos.

Relativamente à acilação, os heterósidos flavonólicos são acilados com ácidos hidroxicinâmicos (ácidos *p*-cumárico, ferúlico, cafeico, sinápico e metoxicafeico) nos açúcares substituintes do hidroxilo no carbono 3, sendo que apenas os substituintes contendo 2 ou 3 açúcares (soforoses e soforotrioses) são acilados. Nas sementes, os dois heterósidos flavonólicos são monoacilados com o ácido sinápico. Na folha interna foram identificados 4 compostos monoacilados com os ácidos sinápico, ferúlico, *p*-cumárico e cafeico. Nas folhas externas e nas inflorescências, foram identificados 3 compostos monoacilados e 3 compostos diacilados, sendo o ácido cafeico comum aos compostos diacilados. Nos rebentos caulinares todos os compostos com resíduos de soforotriose em 3 são acilados e os 2 únicos compostos com resíduos de soforose são não acilados. Assim, foram identificados 12 compostos monoacilados e 6 compostos diacilados com o ácido sinápico em comum nos compostos diacilados. Na larva de *P. brassicae* foram identificados 12 compostos monoacilados e 2 compostos diacilados tendo em comum o ácido sinápico. Em todas as matrizes os compostos diacilados identificados (sempre no carbono 3) são dissubstituídos (nos carbonos 3 e 7).

Os derivados de quercetina são minoritários, principalmente nas amostras obtidas em campo e apenas foram identificados em 3 matrizes: folha interna (1 composto); rebentos caulinares (4 compostos) e larva de *P. brassicae* (5 compostos, sendo um deles comum à folha interna e outro comum aos rebentos caulinares). Estes compostos têm um padrão de substituição semelhante ao já descrito para o campferol. Nos sistemas de cultura *in vitro*, particularmente nos rebentos caulinares, a biossíntese deste flavonol parece induzida, o que se pode ficar a dever à suplementação de fitoreguladores utilizada neste tipo de cultura. As larvas de *P. brassicae* alimentadas com couve proveniente da agricultura são a matriz mais rica em quercetina, o que pode dever-se à sua bioacumulação preferencial ou à biotransformação do campferol em quercetina por oxidases presentes na larva.

Comparando o perfil de flavonóis de todas as matrizes verificou-se que dos 51 compostos identificados nenhum é comum a todas elas. A tendência geral é para cada matriz ter os seus compostos característicos, com excepção das inflorescências que apresentaram o mesmo perfil que as folhas externas e das plântulas que exibiram o mesmo perfil de compostos das sementes. As restantes matrizes têm apenas alguns compostos comuns, principalmente alguns dos flavonóis não acilados. As sementes têm um composto comum com os rebentos caulinares, a folha interna tem 3 compostos

comuns com outras matrizes, a folha externa tem 4, os rebentos caulinares têm 4 e a larva tem 7 compostos comuns com outras matrizes. Dos compostos comuns, o campferol 3-*O*-soforósido foi encontrado na folha interna, folha externa, inflorescências, rebentos caulinares e larva de *P. brassicae*; o campferol 3-*O*-soforósido-7-*O*-glucósido, nas folhas internas, folhas externas, inflorescências e larva de *P. brassicae*; a quercetina 3-*O*-soforósido-7-*O*-glucósido na folha interna e na larva de *P. brassicae*; a quercetina-3-*O*-soforósido nos rebentos caulinares e na larva; o campferol-3-(sinapoil)soforotriósido-7-glucósido nas sementes e nos rebentos caulinares; o campferol 3-*O*-(feruloil)-soforotriósido na folha externa e nos rebentos caulinares e o campferol 3-*O*-glucósido na folha externa e na larva de *P. brassicae*. Na Tabela 9 encontram-se dados que permitem caracterizar os flavonóis de cada uma das matrizes estudadas.

5.1.1.2. Derivados de ácidos hidroxicinâmicos

Os ácidos hidroxicinâmicos são abundantes nas matrizes estudadas, não apenas como grupos acilo de heterósidos flavonólicos, mas também esterificados com glucose, ácido quínico e também com colina.

Os heterósidos não flavonólicos de ácidos hidroxicinâmicos apenas fazem parte do perfil fenólico das sementes, plântulas, folhas internas e rebentos caulinares. Nesta classe foram identificados 22 compostos diferentes, todos eles contendo ácido sinápico, e havendo alguns isómeros. Os compostos têm entre 1 e 3 resíduos de ácidos que podem ser iguais ou diferentes. Para além do ácido sinápico alguns compostos têm ácido ferúlico, cafeico e metoxicafeico. A fracção glicosídica é quase sempre constituída por 1 ou 2 resíduos de glucose com ligações interglicosídicas 1 $\rightarrow$ 6 (genciobioses).

Tal como se verificou para os heterósidos flavonólicos, os derivados não flavonólicos de ácidos hidroxicinâmicos são característicos de cada matriz, havendo apenas alguns compostos comuns às várias matrizes. Destes, um isómero de 1,2-disinapoilgenciobiósido e um isómero de 1,2,2'-trisinapoilgenciobiósido encontram-se em todas as matrizes; 2 outros isómeros de 1,2-disinapoilgenciobiósido encontram-se nas sementes e na folha interna e dois compostos de ácido sinápico e ácido ferúlico, (o 1-sinapoil-2-feruloilgenciobiósido e o 1,2'-disinapoil-2-feruloilgenciobiósido) encontram-se na folha interna e nos rebentos caulinares.

A sinapoilcolina, um marcador taxonómico do género *Brassica*, foi identificada nas sementes de couve tronchuda, tendo-se verificado a sua diminuição nos primeiros dias de germinação da semente e não se detectando a sua presença nas folhas, inflorescências e restantes matrizes estudadas. Sabe-se que a hidrólise da sinapoilcolina

origina trimetilamina conferindo um sabor desagradável (68), mas esta não foi identificada nas partes edíveis da couve tronchuda.

A Tabela 9 contém dados relativos ao tipo de derivados de ácidos hidroxicinâmicos identificados em cada matriz.

Tabela 9. Tipo de compostos polifenólicos encontrados em cada matriz.

	S	P	FI	FE	Inf.	RC	L
Compostos Polifenólicos	13	12	17	15	14	37	20
			Heterósidos Flavonólicos (51)				
Total	2	1	7	14	13	20	20
Quercetina			1	0	0	4	5
Monosubstituídos (3)			1	6	5	7	9
dissubstituídos (3, 7)	1	1	6	8	8	13	11
Trisubstituídos (3, 7, 4')	1						
Monoacilados	2	1	4	3	3	12	12
Diacilados				3	3	6	3
			Ácidos hidroxicinâmicos (22)				
Total	11[a]	11[a]	8[b]			11	
Monoacilados	6[a]	6[a]	2[b]			1	
Diacilados	4	4	4			4	
Triacilados	1	1	2			6	
			Ácidos Clorogénicos (6)				
Total			2	1	1	6	
Diacilados						1	

S: Semente; P: Plântulas; FI: Folha interna; FE: Folha externa; Inf: Inflorescências; RC: Rebentos caulinares, L: larva de *P. brassicae*; a. incluindo 1 éster de colina; b. incluindo o ácido sinápico livre.

De todas as amostras de *B. oleracea* var. *costata* estudadas, a presença de ácidos clorogénicos nos extractos é particularmente importante nos rebentos caulinares. Nesta matriz, para além do ácido 3-*p*-cumaroilquínico também identificado na folha interna e nas inflorescências e do ácido 4-*p*-cumaroilquinico presente nas folhas internas, foram identificados os ácidos 3-cafeoilquínico, 3-feruloilquínico, 4-cafeoilquínico e ainda um composto diacilado, o ácido cafeoilferuloilquínico. Nos extractos de semente e de larva de *P. brassicae* não foram identificados ácidos clorogénicos.

Em resumo, nas sementes foram identificados 13 compostos polifenólicos diferentes, tendo em comum a presença de ácido sinápico, o único ácido hidroxicinâmico identificado nesta matriz. Durante o processo de germinação das sementes verificou-se uma diminuição de todos os compostos fenólicos e não se verificou o aparecimento de nenhum composto novo [4.2, (205)]. Na folha interna foram identificados 17 compostos polifenólicos. Em comparação com as sementes identificaram-se mais derivados do campferol havendo um equilíbrio entre a quantidade de derivados do campferol (7 compostos) e a quantidade de derivados de ácidos hidroxicinâmicos (8 compostos). Nesta matriz, para além do ácido sinápico identificaram-se outros ácidos hidroxicinâmicos, como o ácido *p*-cumárico, cafeico e ferúlico. Aparentemente, as folhas jovens estão muito activas na biossíntese de ácidos fenólicos, encontrando-se grandes quantidades de ácido *p*-cumárico (o primeiro ácido fenólico na via biossintética a partir da fenilalanina) e também de ácido sinápico, que já existia em grandes quantidades na semente. A presença de compostos contendo os ácidos ferúlico e cafeico é minoritária. O ácido *p*-cumárico encontra-se sob duas formas: como grupo acilo de heterósidos de campferol ou esterificado com o ácido quínico. O ácido ferúlico encontra-se esterificado com genciobiose e com heterósidos de campferol, enquanto que o ácido cafeico aparece como grupo acilo de um heterósido de campferol. Nas folhas externas e inflorescências foram identificados 15 compostos polifenólicos. Estas matrizes caracterizam-se pela presença de flavonóides di e triacilados, enquanto que nas sementes e nas folhas internas apenas foram identificados campferóis monoacilados. O grau de glicosilação também vai aumentando. Relativamente ao número de açúcares, nas sementes e na folha interna só se identificaram compostos em que o substituinte do hidroxilo no carbono 7 é a glucose, enquanto que na folha externa aparecem compostos substituídos com soforose nesta posição. No carbono 3 existem fracções glicosídicas contendo 1 a 4 resíduos de glucose. Nas folhas externas e nas inflorescências não se detectaram ésteres de glucose ou de genciobiose com ácido sinápico. Para além da presença de ácido 3-*p*-cumaroilquínico, os restantes ácidos hidroxicinâmicos, cafeico, metoxicafeico, ferúlico e sinápico encontram-se ligados ao campferol. Nos rebentos caulinares

identificou-se uma grande variedade de compostos polifenólicos, 37, com estruturas semelhantes aos compostos identificados nas outras matrizes anteriormente estudadas. Assim, encontraram-se muitos heterósidos de ácidos hidroxicinâmicos não flavonólicos, tal como nas sementes e nas folhas internas e heterósidos flavonólicos com um número elevado de resíduos de açúcar e de ácidos hidroxicinâmicos, tal como nas folhas externas e nas inflorescências. Também foi nesta matriz que se identificou a maior variedade de ácidos clorogénicos (6) e de derivados de quercetina (4). Nesta matriz existem todas as geninas de ácidos hidroxicinâmicos (ácidos *p*-cumárico, cafeico, metoxicafeico, ferúlico e sinápico) e flavonóis (campferol e quercetina) já encontrados nas outras matrizes e foi a única matriz em que se identificaram heterósidos com ramnose. Na larva de *P. brassicae* identificaram-se 20 compostos polifenólicos com um perfil semelhante ao da folha externa que lhe serve de alimento, mas com um maior número de derivados de quercetina (5).

5.1.2. Algumas considerações sobre os cromatogramas obtidos

Os cromatogramas obtidos em LC-DAD de couve tronchuda são muito complexos não sendo fácil obter boas resoluções para todos os compostos. Nas matrizes contendo simultaneamente heterósidos de ácidos hidroxicinâmicos flavonólicos e não flavonólicos, verifica-se que de uma maneira geral os heterósidos flavonólicos eluem primeiro do que os não flavonólicos.

O comportamento cromatográfico dos heterósidos flavonólicos mostra que, em LC de fase reversa, os compostos com maior grau de glicosilação têm um tempo de retenção mais baixo. A posição da glicosilação no núcleo do flavonóide afecta significativamente o tempo de retenção (133). Regra geral, a introdução de uma glucose no hidroxilo no carbono 7 reduz significativamente o tempo de retenção dos compostos glicosilados no carbono 3. Contudo, a introdução de um segundo resíduo de hexose no carbono 7 aumenta o tempo de retenção (104).

A ordem de eluição dos derivados acilados, para o mesmo tipo de substituição glicosídica, não coincide com a dos ácidos livres. Os tempos de retenção dos ácidos livres aumentam pela seguinte ordem: cafeico < ferúlico < sinápico, enquanto que nos flavonóides acilados a ordem é cafeoílo < sinapoílo < feruloílo [4.14, (145)].

A acilação com ácidos hidroxicinâmicos afecta a mobilidade cromatográfica de forma diversa, dependendo da substituição glicosídica do flavonóide. Por exemplo, verifica-se que os compostos acilados sem glicosilação no carbono 7 têm tempos de retenção similares ou superiores aos compostos desacilados correspondentes. Todos os

compostos que se encontram glicosilados no carbono 7 originam compostos desacilados, com tempos de retenção inferiores aos compostos acilados originais (104).

Verificaram-se comportamentos cromatográficos anómalos em vários compostos: há compostos acilados que eluem primeiro que compostos não acilados dos quais derivam; há compostos acilados no carbono 3 do açúcar e sem glicosilação no carbono 7 com tempos de retenção inferiores a alguns compostos glicosilados nas posições 3 e 7 e ainda há isómeros acilados em que um elui antes e outro depois do composto não acilado que lhes deu origem. Este último comportamento parece indicar que a posição em que o açúcar é acilado também influencia a ordem de eluição [4.16, (144)].

O tipo de ligação entre o açúcar e o ácido hidroxicinâmico (éster ou outra), parece afectar o tempo de retenção [4.5, (11)].

5.1.3. Quantificação de Compostos Polifenólicos

Neste trabalho, a caracterização e quantificação dos compostos polifenólicos de várias matrizes de couve tronchuda foi quase sempre realizada em extractos aquosos, obtidos por decocção dos tecidos em estudo. Como o método extractivo afecta não só o tipo de compostos como a quantidade em que cada um deles é extraído, este procedimento permitiu avaliar a quantidade de compostos polifenólicos disponível na forma de consumo habitual da planta, nomeadamente de folhas e inflorescências. Dado que a composição de compostos polifenólicos não integra as tabelas de composição dos alimentos, os valores aqui encontrados podem servir de referência para os cálculos de fenóis ingeridos quando se consome couve tronchuda.

Em trabalhos realizados por outros grupos verificou-se que durante o processo de cozedura, uma parte dos flavonóides são retidos no tecido vegetal, mas a maior parte é libertada para a água de cozedura (101, 113). No primeiro trabalho referido, realizado com brócolos, apenas 14-28% dos heterósidos foram retidos no tecido, sendo os restantes lixiviados para a água de cozedura, havendo ainda uma pequena parte que deu origem às geninas. A quantidade de flavonóis lixiviados depende do tecido sendo maior nas folhas que apresentam uma grande área superficial. Para minimizar este problema todos os tecidos foram reduzidos a partículas de tamanhos semelhantes.

A quantificação foi realizada usando um detector de díodos. Para isso adquiriram-se espectros na gama de 240-400 nm, sendo os cromatogramas registados a 320 nm para quantificar os ácidos hidroxicinâmicos, a 330 nm para quantificar os flavonóis acilados e a 340 nm para quantificar os heterósidos e geninas (103). A selecção do comprimento de onda óptimo para efectuar a quantificação permite obter limites de

detecção de 1-10 ng (134), sendo as perdas, causadas quando se utilizam comprimentos de onda próximos do óptimo, relativamente modestas.

A quantidade de compostos polifenólicos dos extractos aquosos expressos em g/kg peso seco de extracto foi de: 5,9 – 8,5 g/kg na semente [(4.1, 4.2); (143, 205)]; 2,3 – 13,7 g/kg nas plântulas (com diferentes dias de germinação) [4.2, (205)]; 1,4 – 26,0 g/kg na folha interna [(4.3, 4.6); (135, 206)]; 16,0 – 30,4 g/kg na folha externa [4.6, (206)]; 1,7 – 19,0 g/kg nos rebentos caulinares (produzidos com diferentes meios e fitoreguladores) [4.15, (207)]. Para os extractos de inflorescências e de larvas de *P. brassicae* apenas foram analisadas amostras de numa colheita, tendo-se obtido o valor de 19,6 g/kg de compostos polifenólicos para as inflorescências [4.12, (146)]; e 1,9 g/kg para a larva de *P. brassicae* [4.17, (208)].

Em termos quantitativos, cada matriz de couve tronchuda apresenta uma grande variação na quantidade de compostos fenólicos. Para esta variação, contribui além do tipo de matriz, o tipo de fertilização, o stress ambiental, nomeadamente a existência de pragas como a larva de *P. brassicae*, a época da colheita, o processo de extracção e a manipulação (separação da parte edível da parte não edível; separação entre folhas internas e folhas externas) (125). Nas amostras de couve tronchuda produzidas em campo, o controlo de alguns destes factores, como o stress ambiental decorrente da falta de água, poluição e ataque de herbívoros é dificultado e a sua contribuição para as variações encontradas na quantidade de compostos fenólicos não pode ser avaliada.

Uma das qualidades atribuídas à couve tronchuda é a grande resistência a condições climatéricas adversas, que tornam possível a colheita durante os meses de inverno. A influência da época da colheita na quantidade de compostos fenólicos não é fácil de prever. Por um lado, a exposição solar parece contribuir para o aumento dos compostos fenólicos, o que pode ser relacionado com a sua função de filtros solares [4.6, (206)]. Por outro lado, a existência de temperaturas muito baixas também pode induzir a biossíntese de heterósidos flavonólicos para evitar a congelação da água em meses muito frios (com períodos solares curtos). Por estas razões, nas folhas não foi possível estabelecer uma correspondência directa entre a quantidade de compostos fenólicos e a época de colheita [(4.5, 4.4); (11, 209)].

Além de resistente ao frio, a couve tronchuda é nutricionalmente pouco exigente. Nos vários ensaios de fertilização realizados, verificou-se que as couves cultivadas sem qualquer fertilização ou em regime de agricultura biológica tem um menor desenvolvimento em termos de produção de biomassa, mas uma maior quantidade de compostos polifenólicos, sendo estas diferenças mais acentuadas para a folha interna. O desenvolvimento das couves foi maior quando se utilizou fertilização convencional com

níveis moderados de fertilizantes, não sendo incrementado com níveis mais elevados ou com nutrientes adicionais como o boro e o enxofre [4.6, (206)].

Num dos trabalhos, a quantificação dos compostos fenólicos em 8 amostras de folha interna foi feita em extractos metanólicos [4.4, (209)]. Comparando os valores obtidos neste trabalho com os obtidos com os extractos aquosos [4.6, (206)] pode concluir-se que a quantidade de compostos polifenólicos é consistentemente e significativamente mais baixa nos extractos metanólicos, o que pode ser explicado pelo elevado grau de glicosilação típico dos compostos encontrados na couve tronchuda. Além disso, no extracto metanólico, a proporção entre derivados de ácidos hidroxicinâmicos e heterósidos flavonólicos é tendencialmente maior do que nos extractos aquosos. Particularmente o composto campferol 3-*O*-soforósido-7-*O*-glucósido, que é o composto predominante na maior parte dos extractos aquosos de folha interna, não se encontra ou encontra-se em quantidades vestigiais nos extractos metanólicos.

5.1.4. Considerações sobre a produção de compostos fenólicos dos rebentos caulinares de couve tronchuda

Nos sistemas de micropropagação tal como no cultivo em campo, o desenvolvimento das plantas e a produção de compostos fenólicos é condicionado por um número de factores complexos, como a constituição genética, a disponibilidade de água, de nutrientes (macro e micro nutrientes) e de reguladores de crescimento. O desenvolvimento das plantas também é condicionado por factores físicos como a luz, temperatura, pH e concentração de oxigénio e de dióxido de carbono (210).

Nos sistemas de micropropagação os nutrientes, incluindo os sais inorgânicos, compostos orgânicos, complexos naturais e suportes inertes são fornecidos pelos meios de cultura. Na realização deste trabalho utilizaram-se duas formulações padronizadas, os meios MSM (*Murashige and Skoog basal medium*) e B5 (*Gamborg's B5 media)*, tendo-se verificado que com o meio MSM se obtêm rebentos caulinares com uma maior quantidade de compostos polifenólicos. Também se verificou que, usando a mesma suplementação do meio, a produção de compostos polifenólicos é maior quando o meio é usado na forma líquida, o que pode ser justificado pela maior facilidade de absorção dos nutrientes.

Vários estudos previamente realizados por outros autores demonstraram que para o desenvolvimento de rebentos caulinares de *B. oleracea* os meios devem ser suplementados com auxinas e citocininas (211, 212). Na produção de rebentos caulinares de couve-flor o meio foi suplementado com 2 citocininas (3,0 mg/L de 6-

benzilaminopurina e 0,25 de “thidiazuron”) e com a auxina NAA (0,5 mg/L) (211), e na produção de rebentos caulinares de brócolos foi usada a citocinina BA (0,5 mg/L) e a auxina NAA (0,5 mg/L) (212).

Na produção de rebentos caulinares de couve tronchuda com meio MS líquido usaram-se os fitoreguladores ácido 1-naftaleneacético (NAA), uma auxina sintética, na concentração de 0,1 mg/L e a citocinina BAP na concentração de 2 mg/L, com um efeito positivo na produção de compostos polifenólicos.

Nas culturas de plantas do género *Brassica* é muito importante a existência de inibidores de etileno, como por exemplo o nitrato de prata (121). Está descrito que os rebentos caulinares de couve-flor produzidos na presença de nitrato de prata mantêm-se verdes durante 3 semanas, mas na ausência deste agente ficam amarelos ou castanhos e acabam por morrer (211). Contudo, nos trabalhos realizados no âmbito desta dissertação verificou-se que o nitrato de prata diminui a quantidade de compostos polifenólicos produzida pelos rebentos caulinares de couve tronchuda o que pode estar relacionado com a diminuição do stress causado pelo etileno. Pode também haver um maior crescimento vegetativo, com o correspondente aumento do metabolismo primário e diminuição do metabolismo secundário. A mesma razão pode justificar a diminuição na quantidade de compostos polifenólicos produzidos quando se utiliza o ácido giberélico, que é um factor de crescimento.

Assim das condições estudadas, o meio MS líquido suplementado com BAP e NAA revelou-se o mais interessante para a produção de compostos fenólicos.

Os factores físicos como a luz (intensidade, qualidade e duração) e a temperatura são facilmente controlados de forma a obter as condições óptimas de produção. O controlo da luz é muito importante, não só porque está relacionada com o período fotossintético, mas também porque as radiações, principalmente as radiações UV, induzem a produção de compostos fenólicos.

Outro factor que pode influenciar a produção de compostos fenólicos é a esterilização. A eliminação da microflora, necessária para que haja o crescimento da planta, pode diminuir a produção de compostos fenólicos usados na interacção planta - microrganismo. Talvez por estas razões a produção de compostos fenólicos na maior parte das condições utilizadas não foi muito elevada, geralmente inferior a 10 mg/kg de peso seco. Obteve-se uma maior quantidade de compostos fenólicos utilizando o meio MSM líquido com 2 mg/L de BAP e 0,1 mg/L de NAA (19 g/kg de peso seco) e utilizando o meio MSM sólido com 2mg/L de 2,4D (12 g/kg de peso seco).

Como já foi anteriormente referido, a principal característica do perfil fenólico dos rebentos caulinares é o grande número de compostos fenólicos identificados, quando comparado com a couve tronchuda produzida em campo. Contudo, a quantidade de

compostos polifenólicos produzidos atingiu valores superiores noutras matrizes, sobretudo mas folhas externas da couve.

5.2. Perfil de ácidos orgânicos

Neste trabalho procedeu-se à caracterização em ácidos orgânicos de extractos aquosos de várias matrizes de couve tronchuda incluindo as sementes [4.1, (143)], as plântulas [4.2, (205)], as folhas internas [(4.3, 4.4, 4.7); (135, 209, 213)], as folhas externas [(4.4, 4.7, 4.8); (209, 213, 214)] e as inflorescências [4.12, (146)].

O perfil de ácidos orgânicos encontrado em todas as matrizes é muito semelhante, embora existam algumas diferenças quantitativas.

As várias matrizes caracterizam-se pela presença de grandes quantidades de dois ácidos orgânicos, o cítrico e o málico, conhecidos por serem regularmente acumulados nas plantas (26). Estes dois ácidos, presentes nos extractos liofilizados em quantidades da ordem dos g/kg, representam, na maior parte das amostras, mais de 90% dos ácidos identificados. Sabe-se que a quantidade acumulada é afectada por vários factores, tais como o tipo de fixação de carbono, as actividades catabólicas e o estado nutricional da planta (26). Outros factores, como a idade da planta e o tipo de tecido condicionam o perfil em ácidos orgânicos, resultando na predominância de alguns ácidos sobre outros (215). De uma forma geral, nas folhas internas e nas plântulas com menos dias de germinação, existe uma tendência para o ácido cítrico ser o composto maioritário, enquanto que nas folhas externas e nas plântulas com mais tempo de germinação o ácido málico tende a ser o composto maioritário. Em geral, as folhas externas e as plântulas com mais tempo de germinação têm maior quantidade de ácidos orgânicos. Também se verifica que a couve tronchuda produzida no regime de fertilização convencional tem maior desenvolvimento de biomassa e maiores teores de ácidos orgânicos.

Os ácidos málico e cítrico desempenham variadas funções nas plantas, o que justifica a sua acumulação. O ácido málico é um composto versátil, que pode ser facilmente transportado através das membranas que separam os vários compartimentos ou então ser armazenado nos vacúolos. Para além de ser utilizado como substrato para a produção de adenosina 5'-trifosfato (ATP), o ácido málico serve para manter o pH citosólico e actua ainda como agente osmótico e antagonizador dos iões potássio e sódio (20, 215, 216). O ácido cítrico desempenha um papel importante na translocação do ferro nas raízes e no transporte de longa distância para as folhas através do xilema, além de ter actividade antioxidante (217).

Para além dos ácidos málico e cítrico, os ácidos aconítico, xiquímico e fumárico são comuns a todas as amostras, estando presentes em quantidades da ordem dos mg/kg de extracto liofilizado.

Os ácidos aconítico (de que se encontram os isómeros *cis* e *trans*) e fumárico são produzidos na mitocôndria pelo ciclo de Krebs, e em menor extensão no glioxisoma, como parte do ciclo glioxilato (218). Dada a natureza catalítica do ciclo de Krebs, estes ácidos orgânicos encontram-se presentes em pequenas quantidades. O ácido aconítico faz parte integrante da biossíntese dos hidratos de carbono e apresenta actividade alelopática (219). O ácido fumárico pode ser metabolizado originando energia e esqueletos de carbono para a produção de outros compostos e pode ainda ajudar a manter o pH celular e a pressão de turgescência (220).

O ácido xiquímico é sempre um composto minoritário nas amostras analisadas. Este ácido e também o ácido quínico, identificado em algumas matrizes, são precursores de compostos aromáticos nas plantas. Particularmente o ácido xiquímico é um precursor dos aminoácidos aromáticos fenilalanina, tirosina e também do triptofano (221).

Em algumas das matrizes analisadas foi possível identificar o ácido oxálico. A quantificação do ácido oxálico é dificultada pelo facto deste ácido co-eluir com o pico do solvente, nas condições de análise utilizadas. O ácido oxálico é o ácido dicarboxílico mais simples. A síntese e acumulação intracelular de ácido oxálico nas plantas estão implicadas na homeostasia celular do cálcio (222). As plantas que crescem em solos alcalinos, em que o cálcio é abundante, muitas vezes reduzem o excesso de cálcio celular combinando-o com ácido oxálico. Isto é essencial uma vez que concentrações elevadas de cálcio interferem com processos celulares essenciais, como a sinalização dependente do cálcio, metabolismo baseado nos fosfatos e dinâmica do citoesqueleto. A precipitação de cristais de oxalato de cálcio pode ser observada em várias espécies de plantas (223). Do ponto de vista nutricional o ácido oxálico é considerado um anti-nutriente, quando ingerido em grandes quantidades, uma vez que diminui a biodisponibilidade do cálcio e, por vezes, de outros minerais (224). Num trabalho realizado com uma variedade de couve-galega e com espinafres verificou-se que a absorção do cálcio a partir da couve por 11 mulheres saudáveis foi superior à absorção a partir dos espinafres, o que foi justificado pelos níveis de ácido oxálico comparativamente mais baixos na couve (225).

Além dos ácidos anteriormente descritos, nas plântulas e nas inflorescências foi identificado o ácido pirúvico.

A quantidade total de ácidos orgânicos (sem quantificar o ácido ascórbico) nos extractos aquosos das diferentes matrizes, expressos em g/kg de extracto liofilizado, foi de 8,0 – 34,7 nas sementes; 43,1 – 79,8 nos plântulas; 11,1 – 252,8 na folha interna e de

15,2 – 202,5 na folha externa. Nas inflorescências só se ensaiaram amostras obtidas numa colheita tendo-se obtido o valor de 49,2 g/kg de extracto liofilizado.

No decorrer desta dissertação não encontramos referências a trabalhos realizados com o objectivo de obter o perfil de ácidos orgânicos de variedades de couves da espécie *B. oleracea*. Num trabalho realizado com o objectivo de determinar vários nutrientes de uma variedade de couve-galega, foram quantificados os ácidos cítrico e málico respectivamente com 2102 e 143 mg/kg de peso fresco (assumindo uma percentagem de humidade de 90,5%, Tabela 1) (226). Na couve tronchuda o ácido cítrico variou entre 119 e 1630 mg/kg de peso fresco e o ácido málico variou entre 172 e 9048 mg/kg de peso fresco, pelo que não se pode concluir que as duas variedades têm concentrações diferentes destes 2 ácidos. Num outro trabalho realizado com o objectivo de obter a assinatura metabólica de brócolos verificou-se que os ácidos orgânicos; fumárico, málico, 2-oxoglutárico e cítrico são importantes para a descriminação entre variedades das amostras estudadas (227).

Na couve tronchuda, quando se avaliou a influência da fertilização, data da colheita e do tipo de folha na produção de ácidos orgânicos, verificou-se que se pode usar a composição em ácidos orgânicos para discriminar entre as plantas produzidas sem fertilização ou com fertilização orgânica das plantas obtidas com fertilização convencional [4.7, (213)].

Sabe-se que a couve tronchuda é muito rica em ácido ascórbico, apresentando cerca de 580 mg/kg peso fresco como se pode ver na Tabela 1. No presente trabalho o ácido ascórbico foi quantificado nos extractos aquosos de couve tronchuda sendo por vezes o composto maioritário no perfil de ácidos orgânicos. No entanto, este ácido é pouco estável e nas condições experimentais utilizadas é possível que ocorra a sua rápida degradação impedindo uma boa quantificação. Mesmo assim, nas amostras em que foi possível fazer a determinação, os valores obtidos foram próximos do valor tabelado. A quantidade de ácido ascórbico expressa em relação ao peso fresco foi de 338 mg/kg nas sementes, 238 mg/kg a 1430 mg/kg nas folhas internas e 344 mg/kg a 1230 mg/kg na folha externa. Estes valores estão de acordo com valores obtidos com outras variedades de *B. oleracea*. Por exemplo, o ácido ascórbico foi quantificado em 410, 517 e 78 mg/kg de peso fresco em amostras de couve roxa, couve repolho e couve lombarda respectivamente (228). Nos brócolos em diferentes estágios de desenvolvimento, o ácido ascórbico variou entre os 377 e os 1249 mg/kg de peso fresco (115). Num trabalho realizado para avaliar a influência das condições agronómicas na produção de brócolos, o ácido ascórbico variou entre 641 e 1217 mg/kg de peso fresco (229).

5.3. Perfil de aminoácidos livres

Os aminoácidos livres estão envolvidos no metabolismo secundário das plantas, como a biossíntese de compostos fenólicos e glucosinolatos e desempenham directa ou indirectamente um papel importante nas interacções planta-ambiente e na saúde humana (26).

O teor de proteínas das folhas internas e externas de couve tronchuda foi previamente determinado, sendo o seu valor da ordem dos 20 g/kg de peso fresco (12), valor que também está de acordo com as tabelas de composição nutricional (Tabela 1). Embora existam valores tabelados para os aminoácidos proteicos, a composição em aminoácidos livres dos alimentos não se encontra disponível nas tabelas de composição alimentar (230).

Na caracterização de 18 amostras de couve tronchuda, 9 de folha interna e 9 de folha externa, obtidas com fertilização convencional em 3 épocas de colheita, a quantidade de aminoácidos livres nos extractos aquosos variou entre 3,3 e 14,4 g/kg de peso fresco [4.9, (231)]. De uma forma geral as quantidades determinadas na couve tronchuda são muito elevadas, quando comparada com os valores de 20 - 200 mg /kg de peso fresco, descritos para a maior parte das plantas (232). Esta característica pode contribuir significativamente para o valor nutricional da couve tronchuda. Noutras variedades de couve da espécie *B. oleracea*, as quantidades de aminoácidos livres são frequentemente muito elevados embora inferiores aos encontrados para a couve tronchuda (233, 234).

Em termos médios os aminoácidos maioritários nos extractos de couve tronchuda foram a arginina (35,6%), a prolina (16,8%), a treonina (8,9%) e a glutamina (7,1%). Os aminoácidos isoleucina, triptofano, fenilalanina e histidina representam menos de 1% da quantidade de aminoácidos livres na folha externa, enquanto que na folha interna os aminoácidos glicina, valina, isoleucina triptofano e fenilalanina representam cada um deles menos de 1%.

Em contraste com o perfil habitual de aminoácidos livres, em que os ácidos glutâmico e aspártico e as suas amidas ácidas glutamina e asparagina são predominantes nas plantas (26), nos extractos aquosos de couve tronchuda os aminoácidos prolina e arginina nas folhas externas e arginina nas folhas internas são os maioritários. Embora a acumulação destes aminoácidos, particularmente da arginina seja pouco comum, outros autores verificaram que no perfil da couve-galega (aminoácidos presentes nas proteínas e livres) estes dois aminoácidos existem em grandes quantidades (aproximadamente 2,8 e 1,8 g/kg de peso fresco para a arginina e prolina respectivamente) (226). Na couve-galega a prolina também é um dos aminoácidos livres

maioritários representando 13,8 - 23,1% dos aminoácidos livres totais (233). A acumulação de prolina pode ser justificada pela existência de condições de stress durante o cultivo (235). O facto da couve tronchuda acumular arginina, em aminoácido com um grupo substituinte básico, e não os aminoácidos com grupos substituintes ácidos (glutâmico e aspártico), poderá estar relacionada com o tipo de solo.

O perfil qualitativo e quantitativo de aminoácidos livres variou significativamente entre tecidos e com o estado metabólico da planta. Por exemplo as quantidades dos aminoácidos valina, prolina, arginina, leucina, cisteína, lisina, histidina e tirosina são significativamente diferentes nos extractos aquosos de folha interna e de folha externa. Enquanto que a prolina é o aminoácido maioitário nas folhas externas (representando entre 31 e 38% do total dos aminoácidos livres), nas folhas internas não ultrapassa os 4,1% (variou entre 0,2 e 4,1%). Pelo contrário, na folha interna a arginina representou entre 43 e 49% dos aminoácidos livres totais, enquanto que na folha externa variou entre os 18 e os 32%.

Comparando as percentagens de aminoácidos livres essenciais nos extractos de couve tronchuda (valores médios das 3 colheitas) com as percentagens definidas pela WHO para a proteína ideal (Tabela 10) verifica-se que a treonina e a soma da metionina com a cisteína se encontram acima do valor tabelado enquanto que todos os outros aminoácidos livres têm percentagens inferiores aos valores definidos pela WHO. Para estes últimos, os valores encontrados na folha externa são superiores aos encontrados na folha interna.

Tabela 10. Percentagem de aminoácidos essenciais na proteína ideal definida pela WHO em comparação com a percentagem obtida nos extractos de folha externa e folha interna de couve tronchuda (226, 231)

	Tre	Val	Iso	Leu	Tri	Lis	Met+Cis	Fen+Tir
WHO	3,4	3,5	2,8	6,6	1,1	5,8	2,5	6,3
FE	6,1	0,7	0,6	1,6	0,0	3,9	2,6	2,2
FI	12,7	0,1	0,5	0,5	0,2	0,9	5,5	1,3

Tre: Treonina, Val: Valina, Iso: Isoleucina, Leu: Leucina, Tri: Triptofano, Met: Metionina, Cis: Cisteína, Fen: Fenilalanina, Tir: Tirosina. FE: folha externa, FI: folha interna

Fonte: FAO/WHO/UNU. (1985). Energy and protein requirements. Report of a Joint FAO/WHO/UNU Expert Consultation. WHO Tech. Rep. Ser. No. 724. Geneve: WHO.

Na folha interna foi possível quantificar a histidina, um aminoácido não-essencial, que na maior parte das plantas se encontra em quantidades vestigiais.

Comparando os resultados obtidos com outras variedades de couve da espécie *B. oleracea*, verifica-se que há algumas diferenças em termos qualitativos e quantitativos. A quantidade de aminoácidos livres de uma variedade de couve-galega variou entre 1,2 e 5,1 g/kg de peso fresco, em amostras cultivadas com níveis crescentes de azoto (233). Os aminoácidos maioritários foram o ácido glutâmico (33,1%), a prolina (18,3%) e a glutamina (10,6%). No mesmo trabalho as quantidades de aminoácidos livres determinados na couve-flor variaram entre 1,5 e 13,1 g/kg de peso fresco. Para esta variedade de *B. oleracea* os aminoácidos maioritários foram o ácido glutâmico (31,6%), o ácido aspártico (18,7%) e a glutamina (15,6%). Em brócolos a concentração de aminoácidos livres foi determinada em 1,9 g/kg (assumindo 91,9% de humidade, Tabela 1), tendo atingido valores de 4,9 g/kg em plantas obtidas com fertilização de selénio. Os aminoácidos maioritários nas amostras controlo foram o ácido glutâmico (23,8%), a glutamina (17,3%), o ácido aspártico (15,8%) e a serina (13,0%) (234). Gomes e Rosa também verificaram que nos brócolos os aminoácidos maioritários são a glutamina e o ácido glutâmico, sendo que os aminoácidos glicina, leucina, metionina, fenilalanina, treonina, triptofano e tirosina, representam cada um menos de 1% do teor total em aminoácidos (230).

Durante a realização desta dissertação não se determinou a composição de aminoácidos livres, assim como a composição em compostos voláteis dos rebentos caulinares e da larva de *P. brassicae* por falta de amostra. Pela mesma razão nestas matrizes não foram realizados alguns ensaios de actividade antioxidante.

5.4. Compostos Voláteis

Os compostos voláteis e semi-voláteis identificados nos extractos liofilizados de couve tronchuda pertencem a muitos grupos diferentes, incluindo acetais, ácidos gordos, aldeídos, alcoóis, ésteres, cetonas, isotiocianatos, tiocianatos, sulfuretos, terpenóides e norisoprenóides, nitrilos, benzenóides e fenilpropanóides [4.10, (236)].

A maior parte dos estudos existentes da caracterização dos compostos voláteis de variedades de couve da espécie *B. oleracea* têm objectivos muito específicos como a avaliação da resposta aos ataques de insectos incluindo a atracção de parasitóides dos insectos agressores (237) e da agressão mecânica das folhas na libertação dos “voláteis das folhas verdes” (238). Por essa razão a fracção volátil é recolhida com a planta

metabolicamente activa e os resultados apresentados não incluem todos os compostos detectados na fracção volátil, mas apenas os mais importantes para o objectivo de cada trabalho. Alguns dos compostos identificados nesses trabalhos também aparecem no perfil de compostos voláteis dos extractos liofilizados de couve tronchuda nomeadamente alguns dos compostos com origem na oxidação dos ácidos gordos e os terpenóides cíclicos. A caracterização exaustiva dos compostos do aroma foi realizada na couve *B. oleracea* var. *gongylodes*, que não é muito consumida em Portugal (239). Também foi realizado um trabalho com brócolos com o objectivo de avaliar os compostos do aroma com impacto na percepção olfactiva (240).

Os ácidos gordos saturados e insaturados estão na origem da maior parte dos compostos voláteis das plantas, tanto em termos qualitativos como quantitativos. Na couve tronchuda foram identificados 36 compostos com origem provável na oxidação dos ácidos gordos contendo entre 5 e 18 átomos de carbono e classificados de acordo com o grupo funcional como acetais, aldeídos, alcoóis, ésteres e cetonas. Estes compostos são ubíquos nas plantas, sendo formados por três processos básicos, a α-oxidação, a β-oxidação e a via da lipoxigenase (oxidação na cadeia) (37).

A α-oxidação envolve ácidos gordos livres com 12 a 18 átomos de carbono que são enzimáticamente degradados a aldeídos gordos de cadeia longa com (n-1) átomos de carbono e CO_2. A α oxidação dos ácidos gordos nas plantas é catalisada pelas enzimas α-dioxigenase / peroxidase e NAD^+ oxirredutase.

A β-oxidação dos ácidos gordos resulta da remoção sucessiva de unidades com dois carbonos (acetil CoA) (37). Existem muitas enzimas que participam na β-oxidação, mas as mais conhecidas são as acil-CoA oxidases (ACX) (241). Os aldeídos e alcoóis de cadeia curta e média emitidos pelas plantas são provavelmente formados pela redução enzimática dos acil-CoA parentais. Alternativamente, os álcoois podem ser formados pela hidrogenação dos aldeídos mediada pela enzima ADH (álcool desidrogenase), e os aldeídos de cadeia média são intermediários do ciclo de α oxidação a partir de ácidos gordos comuns (37). A formação de ésteres voláteis depende do fornecimento de unidades de acil-CoA originadas durante a β-oxidação e de álcoois. As álcool acil-transferases (AAT) são capazes de combinar vários álcoois e acil CoA's.

Os aldeídos e álcoois voláteis saturados e insaturados com 6 e 9 carbonos podem ser formados pela via da lipooxigenase. Pelo menos 4 enzimas estão envolvidas na via biossintética que levam à sua formação: a lipooxigenase (LOX), a hidroperóxido liase (HPL), a 3Z,2E-enal isomerase e a álcool desidrogenase (ADH). A LOX catalisa a dioxigenação régio e enantio-selectiva de ácidos gordos insaturados (e.g. ácidos linoleico

e α-linolénico) contendo uma ou mais unidades de 1Z,4Z pentadienóicas. A HPL quebra os produtos resultantes da LOX resultando na formação de ω-oxo ácidos e aldeídos C_6 e C_9 voláteis. O grupo carbonilo β,γ-insaturado dos produtos da HPL são susceptíveis de isomerização enzimática (catalisada pela 3Z,2E-enal isomerase) ou não enzimática. Os aldeídos C_6 e C_9 podem ser posteriormente metabolizados pela ADH para formar os álcoois correspondentes (37). Num estudo realizado com couves de Bruxelas, verificou-se que a libertação dos compostos atrás referidos em folhas não danificadas é muito reduzida. Contudo, após agressão mecânica são libertados compostos com 5 e 6 átomos de carbono, conhecidos como "voláteis das folhas verdes" (238).

Nos extractos aquosos de couve tronchuda, além das classes de compostos voláteis comuns às outras plantas, foram identificados vários compostos de enxofre incluindo sulfuretos, isotiocianatos e um tiocianato, característicos das crucíferas e que contribuem para o seu aroma característico. Todos estes compostos de enxofre (assim como um nitrilo que também foi identificado) deverão ter origem na degradação de glucosinolatos.

Sabe-se que, durante o processo de cozedura da couve, a quantidade de glucosinolatos que é lixiviada é muito significativa sobretudo quando as amostras são reduzidas a fragmentos muito pequenos (242). Como neste trabalho os extractos de couve tronchuda foram obtidos por decocção de amostras previamente liofilizadas e pulverizadas, a maior parte dos glucosinolatos e dos seus produtos de degradação deverá estar presente no extracto e por esta razão tentou-se verificar a relação entre os compostos presentes no extracto e os glucosinolatos previamente identificados.

Comparando os isotiocianatos identificados por GC-MS com os glucosinolatos previamente descritos na couve tronchuda (Tabela 3) (12), verificou-se que os isotiocianatos de alilo e de butenilo provenientes da hidrólise dos glucosinolatos alifáticos sinigrina e gluconapina respectivamente, se encontram entre os compostos voláteis, sendo que o isotiocianato de alilo tal como a sinigrina é um composto maioritário. Pelo contrário, nenhum isotiocianato proveniente da hidrólise dos glucosinolatos indólicos (glucobrassicina, metoxiglucobrassicina e neoglucobrassicina), ou os indol-3-carbinóis resultantes do rearranjo destes isotiocianatos instáveis, foi encontrado entre os compostos voláteis. Sabe-se que os compostos indólicos se encontram geralmente em pequenas quantidades na fracção volátil, sendo necessário recorrer a técnicas especiais para os detectar em GC-MS (243). Como era de esperar, não foi encontrado nenhum isotiocianato proveniente da progoitrina, pois estes são instáveis e originam a goitrina (5-oxazolidina) (Figura 9). Também não se detectou o isotiocianato resultante da hidrólise da glucoiberina, o único glucosinolato com enxofre na cadeia lateral identificado na couve tronchuda.

O perfil de glucosinolatos e consequentemente dos seus produtos de degradação difere, não só entre espécies de *Brassica*, como varia substancialmente entre cultivares da mesma espécie e diferentes condições de cultivo. Além disso, a acumulação de glucosinolatos varia entre tecidos e estado de desenvolvimento, quer em termos de concentração como de composição. Regra geral, as folhas jovens e os tecidos reprodutores, como as sementes, contêm as maiores concentrações, enquanto as folhas senescentes têm a menor concentração. Os caules, folhas e raízes têm concentrações intermédias (17). Apesar de neste trabalho se ter feito apenas um estudo qualitativo, envolvendo extractos de folhas internas e de folhas externas de plantas obtidas com diferentes regimes de fertilização, verificou-se que nas folhas internas a quantidade de compostos de enxofre foi superior, e que a fertilização com enxofre também contribui para o aumento desta classe de compostos. Relativamente à fertilização com enxofre o resultado obtido está de acordo com o descrito para uma outra variedade de couve da espécie *B. oleracea*, (*B. oleracea* L. var. *gonglyodes*) (244).

Na caracterização do perfil de compostos voláteis da couve atrás referida verificou-se que cerca de 57% dos compostos voláteis tinham origem na degradação dos glucosinolatos. O composto maioritário nesta variedade, o trissulfureto de dimetilo também foi identificado na couve tronchuda.

Nos extractos de couve tronchuda identificaram-se ainda vários compostos da classe dos terpenóides: 7 monoterpenos (6 monocíclicos, incluindo um éter cíclico e um bicílico) e 1 sesquiterpeno monocíclico. Estes compostos fazem parte dos óleos essenciais, e têm uma distribuição generalizada nas plantas.

A couve tronchuda é caracterizada pela presença de grandes quantidades de β-caroteno (12 mg/kg de peso fresco, Tabela 1), o que pode justificar a presença de 8 norisoprenóides (apocarotenóides contendo entre 9 e 13 carbonos) na fracção volátil dos extractos analisados. Tal como se verificou com outros metabolitos secundários, as plantas obtidas com fertilização química, têm menor quantidade de terpenóides e de norisoprenóides.

Na couve tronchuda foram também identificados 2 fenilpropanóides e 1 benzenóide. Os fenilpropanóides (C_6-C_3) e benzenóides (C_6-C_1) voláteis, derivados da fenilalanina, contribuem para o aroma de muitas plantas e têm um papel importante na comunicação com o meio ambiente. Estes compostos, que se encontram presentes em muitas especiarias, são utilizados desde a antiquidade pelos seres humanos na conservação e aromatização de alimentos e como agentes medicinais.

Um dos fenilpropenos mais comuns é o eugenol. Os passos iniciais da biossíntese do eugenol, desde a L-fenilalanina até à formação do coniferol são comuns à biossíntese das lenhinas [transaminação da L-fenilalanina pela PAL formando-se o ácido cinâmico,

seguida da hidroxilação do ácido cinâmico a ácido p-cumárico pela cinamato 4-hidroxilase (C4H), da hidroxilação do ácido *p*-cumárico a ácido cafeico pela p-cumarato 3-hidroxilase (C3H), da metilação do ácido cafeico a ácido ferúlico pela catecol-*O*-metiltransferase (COMT), da redução do ácido ferúlico a coniferaldeído pela cinamoil CoaA reductase (CCR) e deste a coniferol pela cinamoil álcool desidrogenase (CAD)] (245). O passo seguinte de remoção do oxigénio do carbono C-9 é menos usual, sendo provavelmente realizado por redutases dependentes do NADPH (246).

O 4-etilfenol (C_6-C_2), um composto que confere um odor desagradável, poderá ter como precursor o ácido p-cumárico que sofre uma descarboxilação pela cinamato descarboxilase seguida de uma redução pela vinilfenol reductase (247).

O tolueno, o único benzenóide encontrado, pode ter origem no encurtamento da cadeia lateral do ácido cinâmico em 2 unidades de carbono, o que pode acontecer pela via da β-oxidação ou por uma via não oxidativa (37).

5.5. Caracterização do potencial antioxidante dos extractos de couve tronchuda

A caracterização do perfil metabólico da couve tronchuda permitiu confirmar que esta matriz é muito rica em metabolitos secundários, particularmente flavonóis e ácidos hidroxicinâmicos, potencialmente protectores do stress oxidativo.

Para avaliar este potencial antioxidante os extractos aquosos de várias matrizes de couve tronchuda foram testados em sistemas não celulares, contendo um sistema de geração de espécies reactivas, e em sistemas biológicos. Em sistemas químicos (não celulares) avaliou-se a capacidade dos extractos captarem as espécies reactivas 1,1'-difenil-2-picrilhidrazilo (DPPH), superóxido ($O_2^{\bullet-}$), hidroxilo ($^{\bullet}OH$), ácido hipocloroso (HOCl), óxido nítrico ($^{\bullet}NO$) e peroxinitrito ($HNOO^-$). Também se realizaram ensaios para avaliar a inibição da actividade da enzima xantina oxidase, que contribui para a produção de $O_2^{\bullet-}$.

Como se verificou que os extractos tinham potencial antioxidante nos sistemas químicos, um dos extractos de folha externa foi testado num sistema de cultura celular de hepatócitos primários de rato expostos ao paraquato para induzir stress oxidativo. A capacidade de protecção dos hepatócitos pelo extracto foi avaliada medindo parâmetros de viabilidade celular, parâmetros dependentes do estado redox da célula como o ATP, glutationa e a peroxidação lipídica e também a acção do extracto na via de sinalização do NFkB.

Existem vários estudos de avaliação de actividade antioxidante em variedades de couve da espécie *B. oleracea* (228, 248-252). Em muitos destes estudos a actividade antioxidante é avaliada em sistemas químicos contra radicais que não existem nos sistemas biológicos como o DPPH, o ABTS [2,2'-azinobis-(3-etilbenzotiazoline-6-sulfonato)] e o APPH [2,2'-azobis (2-amidinopropano)] (ORAC assay). Também já foram avaliadas a capacidade de redução do ferro (sujeito a oxidação pelo TPTZ, 2,4,6-tripiridil-*S*-triazina) e a inibição da peroxidação lipídica (num sistema com ácido linoleico e o radical AAPH) por extractos de couve da espécie *B. oleracea*. Nos trabalhos realizados verificou-se sempre que os extractos usados têm potencial antioxidante dependente da concentração e que existe alguma correlação entre a actividade antioxidante e a quantidade de compostos fenólicos existente nos extractos.

Foram também realizados alguns ensaios de avaliação do potencial antioxidante em culturas celulares de extractos de couve da espécie *B. oleracea*. Num desses trabalhos verificou-se que os extractos de brócolos diminuem o stress oxidativo induzido pela APPH em células Hep-G_2, embora não diminuam o dano no DNA induzido pelo H_2O_2 (252). Relativamente à neutralização do radical APPH verificou-se que o resultado obtido no ensaio não-celular foi diferente do resultado obtido no ensaio com células, sendo a capacidade dos extractos de brócolos neutralizarem o radical APPH muito inferior no sistema celular. No mesmo estudo verificou-se que os extractos de brócolos induzem a QR.

Num outro estudo realizado com extractos de *Brassica oleracea* var. *alba* verificou-se que os extractos aumentaram a viabilidade de células IMR32 expostas ao H_2O_2, (avaliada pela capacidade de redução do MTT) (249). }. No mesmo trabalho os extractos também demonstraram ter a capacidade de neutralizar o $^{\bullet}OH$, num sistema não-celular.

Cho e os seus colaboradores, 2006, avaliaram a actividade antioxidante de extractos de brócolos em sistemas químicos e também *in vivo* usando um modelo de ratos diabéticos (248). Nos sistemas químicos, para além da actividade antioxidante dos extractos contra o radical DPPH, verificaram que os extractos tinham actividade antioxidante contra o $^{\bullet}NO$. No modelo *in vivo* verificaram que a administração de um extracto de brócolos aos ratos com diabetes induzida por streptozotocina (ZTC) diminuiu a perda de peso dos animais, diminuiu o aumento de peso do fígado e do rim e também diminuiu os níveis de glucose e de proteínas glicosiladas no soro. O extracto também teve efeitos positivos nos níveis de peroxidação lipídica do plasma, do fígado e do rim dos ratos diabéticos.

5.5.1. Potencial antioxidante da couve tronchuda avaliada através de ensaios químicos

O estudo do potencial antioxidante dos extractos aquosos das várias matrizes de couve tronchuda incluindo as sementes [4.1, (143)], as folhas internas [4.3, (135)], as folhas externas [(4.3, 4.8); (135, 214)], as inflorescências [4.12, (146)], os rebentos caulinares [(4.14, 4.15); (145, 207)] e a larva de *P. Brassicae* [4.17, (208)], foi rastreado utilizando o radical DPPH. Como todas as matrizes demonstraram ter actividade antioxidante dependente da concentração contra este radical, avaliou-se a sua capacidade antioxidante contra espécies reactivas de oxigénio e de cloro biologicamente relevantes. Também se avaliou o potencial antioxidante das sementes, das folhas internas e das folhas externas contra espécies reactivas de azoto [4.16, (253)]. Os resultados obtidos estão sumariados na Tabela 11.

Tabela 11. Actividade antioxidante e inibição da xantina oxidase (expressas em µg/mL) das várias matrizes de couve tronchuda e larva de *P. Brassicae*.

	S	FI	FE	Inf.	RC	L
DPPH (IC_{50})	115	2606	N-678	754	789-111	97
$O_2^{\bullet-}$ (IC_{50})						
X/XO	813	351	N-102	1167	811[a]	600[a]
PMS/NADH/O_2	477	288	N-140	913	100	50[a]
Inibição XO (IC_{50})						760[a]
(IC_{25})	N	1723	N-258	1316		358
$^{\bullet}OH$ (IC_{25})	4	27	223-9	172		6
$^{\bullet}OH$ – asc (IC_{25})	<1	22	(-111)-5	N		47
$^{\bullet}OH$ – EDTA (IC_{25})	N	N	N	N		N
HOCl (IC_{10})	87	485	N-257		N	453
$^{\bullet}NO$ (IC_{50})	356	2228	884			
$ONOO^-$ (IC_{50})	302	1641	707			

S: Semente; FI: Folha interna; FE: Folha externa; Inf: Inflorescências; RC: Rebentos Caulinares, L: Larva de *P. brassicae*; X: xantina; XO: Xantina oxidase; asc: Ácido ascórbico; [a]: Valores interpolados na zona próxima de actividade independente da concentração; Valores negativos: actividade pró-oxidante. Actividade pró-oxidante. N: o valor especificado de actividade antioxidante (10, 25 ou 50%) não é atingido.

Nota: foram ensaiadas 6 amostras de folha externa (excepto para avaliar o IC_{50} de RNS) e 10 amostras de rebentos caulinares obtidos em diferentes condições para avaliar o DPPH.

Como se pode concluir pela análise da Tabela 11, os extractos têm claramente potencial antioxidante nos sistemas testados, sendo que o potencial antioxidante de cada matriz varia com o radical testado. Além disso, o potencial antioxidante da mesma matriz obtida em condições diferentes (e com composição química diferente) também varia para um radical específico, como se pode observar com a folha externa ou com os rebentos caulinares.

De uma forma geral, os extractos demonstraram ter uma actividade antioxidante dependente da concentração, numa gama relativamente larga de concentrações, normalmente na gama de 1-2 mg/mL. Para o radical superoxido a dependência da actividade antioxidante com a concentração da maioria das matrizes estudadas verifica-se até aos 250 µg/mL, e para o radical hidroxilo esta dependência verifica-se para concentrações de extracto inferiores a 200 µg/mL. Contudo, em muitas amostras, a proporcionalidade entre concentração de amostra e neutralização do radical em estudo deixa de se verificar antes de se atingir um valor de 50% da neutralização do radical. Em alguns ensaios também não foi possível determinar os valores de IC_{50} por restrições de solubilidade da amostra.

A matriz com maiores variações no potencial antioxidante foi a folha externa, mas para esta matriz foram ensaiadas mais amostras e amostras obtidas em diferentes condições. Nalguns dos extractos de folha externa analisados a capacidade de captação das espécies reactivas não atingiu os 50%. Além disso, no ensaio do radical hidroxilo sem ácido ascórbico alguns dos extractos de folha externa e de inflorescências ensaiados tiveram uma actividade pró-oxidante, em concentrações inferiores a 200 µg/mL.

5.5.1.1. DPPH

O DPPH é um radical livre orgânico com uma cor violeta intensa, cujo electrão livre é estabilizado por ressonância de toda a molécula. Este radical é usado para verificar a capacidade de um antioxidante doar um átomo de hidrogénio, o que pode ser avaliado pela perda da cor característica.

Comparando o potencial antioxidante dos extractos aquosos das várias matrizes de couve tronchuda contra o radical DPPH verifica-se que as sementes e as larvas de *P. brassicae* são as matrizes com maior actividade, as folhas internas e algumas das amostras de folhas externas têm um baixo potencial de redução deste radical e as inflorescências e os rebentos caulinares têm uma capacidade de redução intermédia.

Relacionando o potencial antioxidante com o perfil de compostos polifenólicos de cada matriz verifica-se que a presença de compostos contendo ácido cafeico parece ser

preponderante para o potencial antioxidante contra o radical DPPH. Sabe-se que nos compostos polifenólicos um dos pré-requisitos que reforça a capacidade antioxidante contra este radical é a existência de um grupo catecol, sendo que nesta classe de compostos a eficiência diminui na seguinte ordem: cafeico > sinápico > ferúlico > *p*-cumárico (254, 255). O campferol, o flavonol maioritário em todas as matrizes, tem as características estruturais mais importantes para ser um bom antioxidante contra o DPPH: presença de um hidroxilo no carbono 4' e no carbono 3 e a dupla ligação no anel C (256). Contudo, como nos extractos de couve tronchuda estudados o campferol se encontra glicosilado no carbono C-3 o potencial antioxidante será menor do que o da genina, uma vez que a substituição no hidroxilo em C-3 por um grupo glicosilo diminui a estabilização por ressonância do radical formado. Mesmo assim, a contribuição dos heterósidos de campferol para a actividade antioxidante total não deve ser desprezada como se pode comprovar com uma das amostras estudadas, a amostra C, cujos resultados foram publicados [4.8, (214)]; esta amostra, com quantidades de compostos fenólicos abaixo do limite de quantificação, foi a única que, nas condições testadas, não atingiu 50% de redução do radical DPPH.

As sementes, caracterizadas pela presença de derivados de ácido sinápico, demonstraram uma excelente capacidade antioxidante relativamente ao radical DPPH, para a qual é provável que os compostos polifenólicos não sejam os principais contribuintes. As sementes contêm a maior quantidade de lípidos de qualquer tecido vegetal, incluindo os maiores níveis de ácidos gordos poli-insaturados, e consequentemente níveis elevados de antioxidantes como os tocoferóis para proteger da oxidação os lípidos armazenados (257). Apesar dos estudos de actividade antioxidante terem sido feitos em extractos aquosos, é possível que alguns destes antioxidantes com acção preferencial na fase lipídica tenham sido extraídos, o que pode explicar a boa actividade dos extractos de sementes contra um radical orgânico como o DPPH.

O potencial antioxidante de todos os extractos estudados de couve tronchuda foi inferior ao descrito num sistema semelhante para o ácido ascórbico com um IC_{50} de 55 µg/mL (258).

5.5.1.2. Anião Superóxido

Comparando o poder oxidante do radical DPPH com o do $O_2^{\bullet -}$, sabe-se que a deslocalização do electrão desemparelhado no radical DPPH o torna mais estável e menos reactivo do que o $O_2^{\bullet -}$ (259). Por esta razão efectuaram-se vários ensaios de

avaliação do potencial antioxidante dos extractos contra o $O_2^{\bullet-}$, tendo-se verificado que este radical também é eficazmente sequestrado pela maioria dos extractos.

Na avaliação do potencial antioxidante dos extractos de couve tronchuda contra o $O_2^{\bullet-}$ usaram-se dois processos para formar o radical, um enzimático e outro não enzimático. No ensaio enzimático, a enzima xantina oxidase (XO) catalisa a oxidação da hipoxantina e da xantina a ácido úrico. Nesta reacção o oxigénio funciona como aceitador de electrões. No ensaio não enzimático o $O_2^{\bullet-}$ formou-se pela reacção do oxigénio com o metassulfato de fenazina (PMS) previamente reduzido pelo NADH. Verificou-se que o potencial antioxidante contra o $O_2^{\bullet-}$ varia com o sistema utilizado para gerar o radical, sendo que no sistema não enzimático (PMS/NADH/O_2) os valores de IC_{50} são tendencialmente melhores. As matrizes com um melhor desempenho foram a larva de *P. brassicae* e os rebentos caulinares, sendo que as folhas internas e algumas amostras de folhas externas também demonstraram ter um bom potencial.

A maior actividade antioxidante do extracto de larva de *P. brassicae* para os radicais DPPH e superóxido (no sistema NADH/PMS/O_2) pode ser explicado pela diferente composição em compostos fenólicos. A larva é mais rica em compostos derivados da quercetina (com um grupo catecol no anel B), que na maior parte dos ensaios tem melhor actividade antioxidante que o campferol, com um só grupo hidroxilo no anel B (256). Além disso, o lúmen intestinal de larvas de insectos Lepidoptera, que se alimentam de plantas ricas em compostos fenólicos, tem níveis elevados de ascorbato e GSH e estes podem contribuir para o potencial antioxidante dos extractos de larva (129).

Na maior parte dos ensaios químicos, a actividade antioxidante avalia-se pela capacidade dos extractos interceptarem espécies reactivas. Contudo, este não é o único mecanismo pelo qual os extractos podem exercer o seu potencial antioxidante. A inibição de enzimas envolvidas na formação de espécies reactivas é um mecanismo indirecto de actividade antioxidante que pode ser avaliado quando se utiliza o sistema X/XO; a diminuição da actividade da XO resulta na diminuição da produção de $O_2^{\bullet-}$ e também de ácido úrico. Neste sistema a actividade antioxidante também pode ser exercida pela captação do $O_2^{\bullet-}$ formado. Relativamente à inibição da XO verifica-se que as amostras contendo maiores quantidades de flavonóis têm a maior capacidade de inibir a enzima. Nestes compostos, os grupos hidroxilo nos carbonos 5 e 7 (e a dupla ligação entre os carbonos 2 e 3 que torna o anel B co-planar por conjugação) são essenciais para a inibição da XO. Sabe-se que o efeito de inibição decai quando o hidroxilo no carbono 7, que é muito importante para a actividade de inibição, se encontra glicosilado, o mesmo acontecendo com a glicosilação do hidroxilo do carbono 3 (260). Isto pode justificar que a

larva, com uma maior proporção de flavonóis não substituídos no carbono 7, tenha sido a única matriz para a qual foi possível calcular o valor de IC_{50}.

5.5.1.3. Radical Hidroxilo

A capacidade de neutralização do $^{\bullet}OH$ foi testada no ensaio denominado de oxidação da desoxirribose. Neste sistema, uma mistura de H_2O_2, Fe^{2+}, EDTA e ácido ascórbico, a pH 7,4, origina o radical que ataca a desoxirribose originando fragmentos, que quando aquecidos na presença de ácido tiobarbitúrico em pH ácido formam um composto que absorve a 532 nm (261-263). A redução do ferro (Fe^{3+}) pelo ácido ascórbico aumenta a taxa de formação de $^{\bullet}OH$.

O potencial dos extractos contra o $^{\bullet}OH$ teve um comportamento diferente do potencial demonstrado contra a maioria dos outros radicais testados. Por um lado, para obter uma captação de 25% do radical gerado as concentrações de extracto necessárias são mais baixas do que as necessárias para os outros radicais. Por outro lado, o aumento da actividade com a concentração só se verifica numa gama estreita de concentrações, sendo a neutralização do radical inferior a 50%. Comparando todas as matrizes estudadas, os extractos de sementes e de rebentos caulinares demonstraram ter um maior potencial antioxidante contra o $^{\bullet}OH$.

Sabe-se que os compostos polifenólicos podem captar os radicais $^{\bullet}OH$. A característica estrutural mais importante para a actividade antioxidante dos compostos polifenólicos contra o $^{\bullet}OH$ é a presença de grupos catecol. Pelo contrário, a presença de grupos carboxilo e a cadeia lateral de 3 carbonos com uma dupla ligação presente nos ácidos hidroxicinâmicos, diminui a actividade antioxidante contra este radical (264). Nas matrizes de couve tronchuda os catecóis estão presentes sobretudo nos heterósidos de ácidos hidroxicinâmicos. Embora a natureza das ligações entre os açúcares e os ácidos hidroxicinâmicos não tenham sido determinadas, é possível que pelo menos para alguns dos heterósidos (flavonólico ou não flavonólico) se forme um éster entre o carboxilo do ácido e o grupo hidroxilo do açúcar, mantendo-se neste caso a estrutura do catecol, necessária para a actividade antioxidante.

A presença, nos extractos, de quantidades elevadas de citrato, um ligando fisiológico do ferro que o torna indisponível para participar na reacção de Fenton, também poderá contribuir para a actividade antioxidante demonstrada pelos extractos (265).

No ensaio realizado sem EDTA as amostras não demonstraram ter potencial antioxidante.

No ensaio realizado na ausência de ascorbato, as matrizes tiveram um comportamento semelhante ao observado no ensaio desenvolvido com ácido ascórbico,

com excepção das inflorescências e da folha externa, para as quais se verificou uma acção pró-oxidante. Contudo, o comportamento observado nos ensaios *in vitro* poderá não corresponder ao que acontece *in vivo*. Apesar de nos ensaios *in vitro* o ácido ascórbico aumentar a formação de radicais hidroxilo em reacções de Fenton na presença de metais de transição, *in vivo*, a presença de várias proteínas que se ligam aos metais, como a transferrina e ferritina, limita a disponibilidade de metais activos para envolvimento em reacções redox (265, 266). Esta situação poderá ser a mesma que ocorre com o consumo destas matrizes.

Muitos flavonóides têm maior potencial antioxidante do que o ácido ascórbico. Isto pode dever-se ao sistema conjugado mais extenso dos flavonóides que permite suportar um electrão desemparelhado, aos dois ou mais grupos hidroxilo reactivos e a menos impedimentos estéricos no local de abstracção (159).

5.5.1.4. Ácido hipocloroso

Os neutrófilos são uma importante fonte de compostos oxidantes e provavelmente contribuem para o dano oxidativo associado a várias doenças em que participam células inflamatórias. Os neutrófilos estimulados geram $O_2^{\bullet-}$ e o seu produto de dismutação, o H_2O_2 e libertam mieloperoxidase. A mieloperoxidase, além de oxidar os substratos clássicos a radicais intermédios, tem a propriedade de converter o H_2O_2 e o cloreto em ácido hipocloroso (HOCl) (151). Em certas circunstâncias, o ácido hipocloroso é o principal oxidante produzido pelos neutrófilos.

O ácido hipocloroso é muito reactivo e participa em reacções de oxidação e de clorinação. As reacções de oxidação têm muitos alvos, particularmente os tióis e os tioésteres. Além destes, compostos como o ascorbato, o urato, os nucleótidos piridínicos e o triptofano também são oxidados pelo ácido hipocloroso embora não tão rapidamente.

As reacções de clorinação biológicas mais comuns envolvem aminas (formação de cloraminas) resíduos tirosilo (originando anéis clorados), lípidos insaturados e colesterol (originando cloridrinas) e resíduos de citosina nos ácidos nucleicos. As reacções de oxidação são mais rápidas do que as reacções de clorinação e por isso são favorecidas em sistemas biológicos, tendendo a ser não específicas (151).

Nenhuma matriz de couve tronchuda demonstrou ter um bom potencial antioxidante contra o ácido hipocloroso**,** e por esta razão apenas são fornecidos valores de IC_{10} para as matrizes ensaiadas. Sabe-se que os grupos SH são mais eficazes a captar o ácido hipocloroso do que os grupos OH (267). Alguns compostos contendo grupos tiol com boa capacidade antioxidante contra o ácido hipocloroso são o ácido desidrolipóico, a glutationa e a cisteína. Destes compostos apenas a cisteína foi

quantificada nas folhas internas e nas folhas externas. Apesar da cisteína ser um dos aminoácidos livres maioritários, atingindo concentrações de 550 µg/kg de peso fresco nas folhas internas, estas amostras não tiveram actividade antioxidante contra o ácido hipocloroso

Os compostos polifenólicos também podem exercer actividade antioxidante contra o HOCl. Nos ácidos hidroxicinâmicos as características mais importantes para esta capacidade são o hidroxilo em posição *para* e a presença de grupos hidroxilo ou metoxilo adicionais (160). Assim, a actividade antioxidante de ácidos hidroxicinâmicos contra o HOCl diminui pela seguinte ordem: ácido cafeico > ácido sinápico > ácido clorogénico ≅ ácido ferúlico > ácido *p*-cumárico (268). As sementes, que contêm bastantes heterósidos de ácidos hidroxicinâmicos, foram a matriz que demonstrou ter um maior potencial contra este radical. Nos flavonóis a característica mais importante para a actividade antioxidante contra o HOCl é o número de grupos hidroxilo substituintes, sendo particularmente importante o hidroxilo no carbono 3, que nos extractos de couve tronchuda se encontra sempre glicosilado, o que pode justificar a pouca actividade antioxidante dos extractos (269). A presença de uma dupla ligação entre os carbonos 2 e 3 e a presença de uma estrutura do tipo catecol no anel B, não parecem influenciar a actividade antioxidante contra o ácido hipocloroso.

5.5.1.5. Óxido nítrico

O radical $^{\bullet}NO$ é uma espécie crítica em muitas funções, incluindo a manutenção da pressão sanguínea devido ao seu efeito vasodilatador, estimulação das defesas do hospedeiro no sistema imunitário, regulação da transmissão neuronal no cérebro, regulação da expressão dos genes, agregação de plaquetas, aprendizagem e memória, função sexual masculina, citotoxicidade e citoprotecção (270). Este radical é um segundo mensageiro de grande difusibilidade que pode desencadear efeitos em locais relativamente distantes do seu local de produção (154).

O $^{\bullet}NO$ é formado endogenamente a partir da L-arginina por uma família de enzimas chamadas sintetases do óxido nítrico (NOS) das quais se conhecem três isoformas. Existem 2 isoformas constitutivas, a nNOS ou NOS I e a eNOS ou NOS III, associadas respectivamente aos tecidos neuronais centrais e periféricos e às células endoteliais vasculares, e a iNOS ou NOS II que pode ser isolada de diversas células após indução por mediadores inflamatórios ou produtos bacterianos (154, 271). O aumento da concentração de $^{\bullet}NO$ em situações patológicas leva à formação de $HNOO^{-}$ e das espécies reactivas que dele derivam (RNS) para níveis potencialmente citotóxicos (153, 272).

Neste trabalho o $^{\bullet}NO$ foi obtido usando nitroprusssiato de sódio (SNP), que em solução liberta espontaneamente e de forma controlada o $^{\bullet}NO$. Na avaliação do potencial captador de $^{\bullet}NO$ verificou-se que todas as matrizes (sementes, folhas internas e folhas externas) possuem actividade de uma forma dependente da concentração. Também se verificou que o ácido sinápico (IC_{50} de 79 µM) e o campferol-3-O-rutinósido (IC_{50} de 567 µM) usados por serem padrões comercialmente disponíveis e poderem representar as classes de compostos fenólicos existentes nos extractos de couve tronchuda, têm um potencial captador do radical $^{\bullet}NO$ superior ao do ácido ascórbico (IC_{50} de 1301 µM) nas condições usadas.

Apesar de a libertação inapropriada do $^{\bullet}NO$ estar relacionada com um grande número de patologias, e por isso ser importante dispor de agentes que modulem a sua actividade, a manipulação de um mediador tão universal pode apresentar alguns riscos, sendo preferível intervir na redução da sua formação (271). Como se verá mais à frente, os extractos de couve tronchuda conseguem inibir a activação do factor de transcrição NFkB e por isso podem ter uma acção sobre a enzima iNOS, diminuindo desta forma a sua actividade em condições patológicas.

5.5.1.6. Peroxinitrito

O $HNOO^-$, formado pela reacção entre o $^{\bullet}NO$ e o $O_2^{\bullet-}$, é uma espécie reactiva mais citotóxica que o $^{\bullet}NO$. A tirosina é particularmente susceptível à nitração pelo $HNOO^-$, originando a 3-nitrotirosina (155). Os resíduos de 3-nitrotirosina são considerados marcadores da formação de $HNOO^-$ *in vivo* (153).

A capacidade antioxidante das amostras testadas contra o $^{\bullet}NO$ também foi avaliada contra o $HNOO^-$ (formado pela reacção em meio ácido entre o H_2O_2 e o NO_2^-) sendo os valores de IC_{50} muito semelhantes aos encontrados para o $^{\bullet}NO$.

O potencial antioxidante dos extractos contra o $HNOO^-$ pode ser explicado pela presença em todas as amostras de ácidos hidroxicinâmicos, na forma de heterósidos ou como grupos acilo do campferol. A semelhança entre os ácidos hidroxicinâmicos e a tirosina pode conduzir à supressão da nitração da tirosina pelo peroxinitrito, sendo os próprios ácidos hidroxicinâmicos nitrados (273). A extensão da interacção do ácido hidroxicinâmico com o peroxinitrito depende da natureza e da posição das substituições do anel aromático. A captação do peroxinitrito pode ocorrer por dois mecanismos: nitração e doação de electrões (Figura 38) (155).

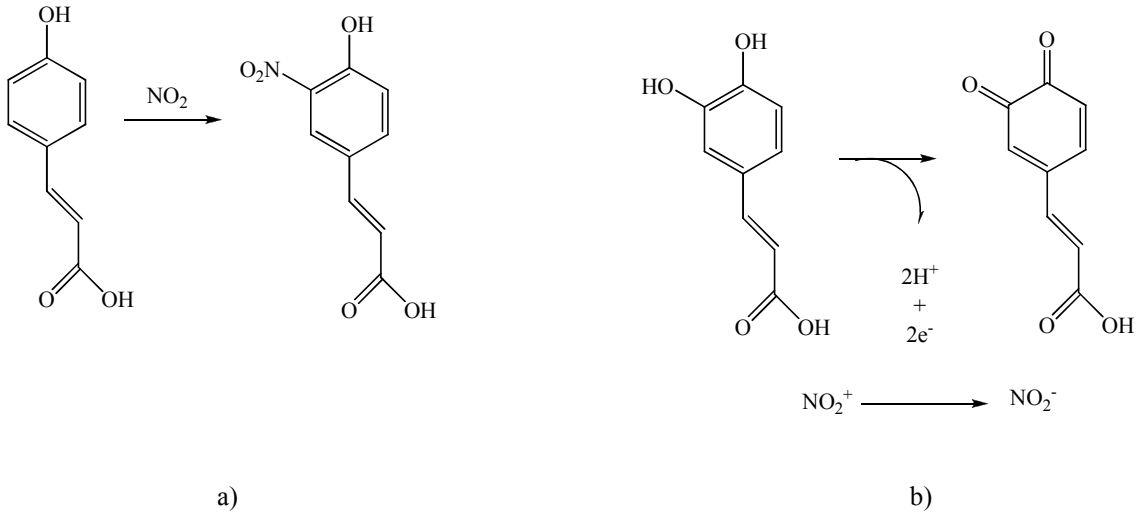

Figura 38. Reacção entre o peroxinitrito e ácidos cinâmicos.

a) Reacção de nitração do ácido p-cumárico. b) Reacção de oxidação do ácido cafeico.

Os monoidroxicinamatos como os ácidos ferúlico e *p*-cumárico são preferencialmente nitrados, protegendo a tirosina de sofrer nitração. Por outro lado a nitração não é observada com os ácidos hidroxicinâmicos contendo grupos catecol (cafeico e clorogénico) pensando-se que deve ocorrer a doação de electrões com a formação de quinonas (155).

No trabalho por nós realizado verificámos que o ácido sinápico (IC_{50} de 39 µM), uma das principais geninas no perfil fenólico da couve tronchuda tem uma capacidade de neutralização do peroxinitrito superior à penicilamina (IC_{50} de 89 µM) a qual é usada como substância de referência na avaliação do potencial antioxidante contra este radical [4.13, (253)].

5.5.2. Avaliação do potencial antioxidante da couve tronchuda em culturas de hepatócitos primários de rato

As culturas de hepatócitos primários são um sistema muito usado no estudo do metabolismo, indução e inibição enzimáticas e nos ensaios de hepatotoxicidade / hepatoprotecção. Trata-se de uma técnica simples, mas versátil, que permite uma manipulação simplificada das células e um controlo rigoroso do ambiente extracelular. Nestes sistemas eliminam-se factores como o fluxo de sangue, heterogeneidade do tipo de célula, factores humorais e nervosos, estado hormonal e nutricional e fornecimento e concentração de oxigénio. O tempo de exposição aos compostos também é mais facilmente controlado (189).

Nos sistemas de cultura celular é importante ter em conta que o ascorbato, flavonóides, e outros compostos polifenólicos e tióis são habitualmente instáveis nos

meios de cultura, facilmente oxidáveis e propensos a gerar artefactos se adicionados em concentrações elevadas (274). Além disso, é geralmente aceite que as células que sobrevivem em cultura nem sempre são representativas das células *in vivo*, em termos de metabolismo, expressão dos genes e níveis enzimáticos, sendo necessário ter algumas reservas para extrapolar os dados obtidos (275).

Apesar destas desvantagens, os sistemas de cultura de células são muito importantes para a realização de estudos mecanísticos. Para confirmar o potencial antioxidante demonstrado pelos extractos de couve tronchuda nos ensaios químicos realizou-se um estudo num sistema celular, utilizando culturas de hepatócitos primários de rato. Os hepatócitos foram incubados com uma das matrizes mais ricas em compostos fenólicos, um extracto de folha externa, produzida em agricultura biológica, contendo 30 g/kg de compostos fenólicos (peso seco). Como na caraterização do perfil de compostos fenólicos se verificou que estes se encontram na forma de heterósidos com um grande número de unidades de glucose, o extracto foi hidrolisado para facilitar a absorção dos compostos fenólicos. Os hepatócitos foram sujeitos a stress oxidativo induzido pelo paraquato (PQ) (10 mM) durante 24 horas. Os efeitos do extracto e do PQ nos hepatócitos foram avaliados por parâmetros de viabilidade celular (MTT e LDH), parâmetros dependentes do estado redox da célula como o ATP, glutationa e a peroxidação lipídica. Também se avaliou a acção do extracto na via de sinalização do NFkB [4.11, (276)].

Como já tinha sido observado em trabalhos anteriores (252), o potencial demonstrado nos ensaios químicos, nem sempre corresponde aos resultados observados em ensaios biológicos. Para a couve tronchuda, os resultados químicos e biológicos foram contraditórios, principalmente para as concentrações mais elevadas de extracto: acima de 800 µg/mL o extracto agravou os efeitos do stress oxidativo induzido nas células pelo PQ como se comprovou pelo aumento da morte celular medido pela diminuição da metabolização do MTT, pelo aumento da libertação de LDH e pela diminuição dos níveis de ATP intracelular. Num ensaio realizado sem células com geração de $O_2^{\bullet-}$ pelo sistema NADH/PMS/O_2 o mesmo extracto demonstrou ter potencial antioxidante em concentrações acima dos 800 µg/mL.

A diminuição da viabilidade celular dos hepatócitos expostos ao PQ e ao extracto dependeu da concentração do extracto até aos 1200 µg/mL, sendo que a partir desta concentração de extracto a viabilidade foi muito reduzida. A diminuição dos níveis de ATP dos hepatócitos expostos ao PQ foi significativamente maior quando os hepatócitos foram expostos a concentrações de extracto superiores a 1600 µg/mL e a diminuição da glutationa total foi significativa para concentrações de extracto acima dos 2400 µg/mL.

Os extractos por si só não demonstraram ter toxicidade para os hepatócitos em concentrações entre os 3,1 µg/mL e os 4000 µg/mL, pelo que se pode concluir que a interacção entre os sistemas antioxidantes endógenos, o sistema de geração de radicais e o extracto resultou em danos adicionais para as células.

Nas células, a eficácia antioxidante dos extractos depende não só da reactividade química contra os radicais, como também da lipofilia, do metabolismo celular, da interacção com outras moléculas e do destino do radical formado a partir do composto antioxidante. A baixa reactividade para alvos intracelulares críticos é um pré-requisito para o potencial antioxidante dos compostos polifenólicos. Pelo contrário, o consumo de agentes redutores como a GSH, o NADH e o NADPH por radicais fenoxilo pode causar efeitos citotóxicos mesmo que o composto polifenólico possa proteger os lipídos ou ácidos nucleicos contra o dano oxidativo (277). A QR, uma enzima que utiliza como co-factores o NADH e o NADPH, pode estar envolvida na destoxificação dos compostos quinónicos formados pela oxidação de flavonóides, como o campferol, presente em grandes quantidades nos extractos de couve tronchuda. A indução da actividade da quinona redutase pelo extracto atingiu os 74% nos hepatócitos expostos a concentrações de extracto de 800 µg/mL (5,6 µM de equivalentes de campferol), o que pode corroborar a hipótese da quinona redutase estar envolvida na destoxificação do campferol ou dos seus metabolitos.

O extracto na concentração 200 µg/mL demonstrou ter algum efeito protector para os hepatóctios expostos ao PQ como se pôde observar pela tendência para diminuir a peroxidação lípidica. Contudo, nesta concentração o extracto reverteu o aumento da actividade da catalase e da activação do NFkB induzida pelo PQ.

Existem algumas evidências de que os efeitos celulares dos flavonóides podem ser mediados por interacções com proteínas específicas centrais nas cascatas de sinalização intracelulares, podendo esta acção ser exercida com concentrações muitas mais baixas do que as necessárias para a actividade antioxidante (161). Por exemplo, os flavonóides podem proteger os neurónios contra o stress oxidativo mais eficazmente que o ascorbato, mesmo na presença de concentrações de ascorbato dez vezes superiores, o que corrobora a hipótese de uma actividade não directamente relacionada com o seu potencial antioxidante (278).

A regulação de genes envolvendo espécies reactivas pode ser feita em diferentes níveis, incluindo factores de transcrição, translacção e ou/estabilidade do mRNA, a regulação da estabilidade proteica e a localização celular das moléculas sinalizadoras (279). }. A alteração da expressão dos genes é uma forma muito eficiente de uma célula responder a sinais extracelulares e/ou alterações no seu ambiente, a curto e a longo termo. Os factores de transcrição desempenham um papel muito importante, pois em

resposta a estes sinais ligam-se a regiões de controlo dos genes alvo e alteram a sua expressão (280).

Os factores de transcrição são substâncias endógenas, habitualmente proteínas, sendo eficientes na iniciação, estimulação, ou finalização do processo genético de transcrição no núcleo, enquanto no citoplasma, o factor de transcrição se encontra numa forma inactiva. O mecanismo de acção de muitos factores de transcrição envolve um evento sinalizador como a modificação da alteração do estado de fosforilação e posterior translocação de uma subunidade para o núcleo. A transdução celular envolve interacções complexas de múltiplas vias celulares (280).

Os factores de transcrição Rel/NFkB estão envolvidos numa vasta gama de processos de controlo, como os processos imunes e inflamatórios, processos de desenvolvimento, crescimento celular e morte celular programada (apoptose). Além disso, estes factores também são importantes em vários estados de doença como o cancro, artrite, inflamação, asma, doenças neurodegenerativas e problemas cardiovasculares (280).

O NFkB existe numa forma latente no citoplasma de células não estimuladas na forma de um dímero transcripcionalmente activo ligado à proteína de inibição IkB. Existem várias subunidades do NFkB incluindo p50, p65 (Rel A), c-Rel, p52 e Rel B, sendo o dímero p50/p65 a forma predominante. Também existem múltiplas formas da proteína IkB (IkBα, β, γ (p105), δ (p100), ε e Bcl-3). Na maior parte dos estudos realizados para determinar o mecanismo de activação do NFkB este encontrava-se associado ao inibidor IkBα. Quando o NFkB é estimulado por muitos indutores o IkBα é rapidamente fosforilado em 2 resíduos de serina (S32 e S36) por cinases do IkB sendo posteriormente degradado pelo proteossoma 26 S. O dímero de NFkB libertado pode deslocar-se para o núcleo e activar os genes alvo ligando-se aos elementos kB dos seus promotores, para os quais tem afinidade (Figura 39).

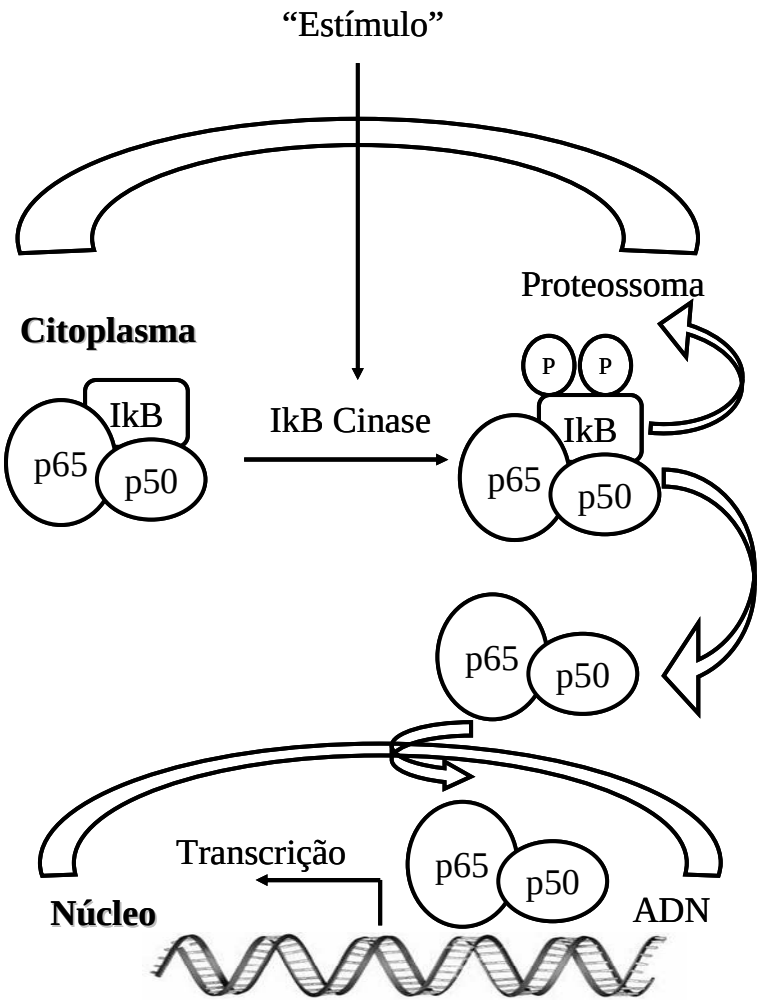

Figura 39. Via de sinalização do NFkB

Uma das explicações possíveis para tantos agentes diferentes activarem o NFkB é o facto de no seu mecanismo de acção estar envolvido o aumento de ROS, e consequentemente do stress oxidativo no interior da célula (281). Como este factor de transcrição responde directamente ao stress oxidativo, a sua acção pode ser inibida por antioxidantes, como os compostos polifenólicos existentes na couve tronchuda.

Neste trabalho verificou-se que a indução da activação do NFkB pelo PQ foi revertida pelo extracto, o que poderá ter resultado num agravamento dos efeitos do stress oxidativo gerado pelo PQ. Como o NFkB está envolvido na expressão de muitos genes de defesa, o extracto poderá ter impedido os hepatócitos de responderem eficazmente à agressão a que estavam sujeitos.

A formação de grandes quantidades de espécies reactivas de oxigénio durante a oxidação dos flavonóides pode induzir a apoptose e a necrose nas células. Contudo, pequenas quantidades de ROS podem resultar numa resposta adaptativa, que permite às

células protegerem-se contra um “ataque” oxidativo subsequente (169). Por outro lado, as propriedades pró-oxidantes dos flavonóides e outros polifenóis têm demonstrado contribuir para a apoptose de células tumorais e para a quimioprevenção do cancro induzida por estes agentes (178).

In vivo, os metabolitos de flavonóides circulantes, nomeadamente as formas glucurónicas e *O*-metiladas e os metabolitos intracelulares, como por exemplo os conjugados de flavonóides com a glutationa, têm uma capacidade reduzida de doar hidrogénio e são menos eficazes na captação de espécies reactivas de oxigénio e de azoto do que as geninas originais (282, 283). Além disso, as concentrações de flavonóides e dos seus metabolitos *in vivo*, por exemplo no plasma ou em órgãos como o cérebro são muitos inferiores aos verificados para outras moléculas antioxidantes com origem na alimentação como o ácido ascórbico e o α-tocoferol. Como consequência, é provável que os efeitos protectores dos flavonóides não se devam à sua capacidade de captar radicais livres. Contudo, os flavonóides podem exercer efeitos directos no tracto gastrointestinal devido às elevadas concentrações aí atingidas. Estes efeitos podem incluir a quelatação do ferro, captura de espécies reactivas e talvez a inibição das ciclooxigenases e das lipooxigenases (284).

5.5.3. Actividade antimicrobiana

A actividade anti-microbiana dos flavonóides tem sido objecto de estudo nos últimos anos, tendo-se verificado que alguns compostos desta classe possuem actividade anti-fúngica, anti-viral e anti-bacteriana (285). A presença de quantidades elevadas de derivados de campferol nos extractos de couve tronchuda justificou a avaliação do seu potencial antimicrobiano.

O potencial antimicrobiano das inflorescências de couve tronchuda foi avaliado contra 3 bactérias Gram + (*B. cereus*, *B. subtilis* e *S. aureus*), 3 bactérias Gram – (*P. aeruginosa*, *E. coli* e *K. peumoniae*) e 2 fungos (*C. albicans* e *C. neoformans*), tendo-se verificado que apenas as bactérias Gram +, sobretudo o *S. aureus* eram inibidos para concentrações de extracto de 100 µg/mL [4.12, (146)].

A utilização de compostos fitoquímicos como agentes antimicrobianos naturais tem-se tornado popular. A principal vantagem da utilização destes compostos como agentes antimicrobianos advém da grande variedade de compostos, o que potencialmente dificulta o fenómeno de resistência desenvolvida pelos micorganismos, como se observa com a utilização a longo prazo dos antibióticos sintéticos. O mecanismo provável de actividade antimicrobiana dos extractos ricos em compostos fenólicos não está bem esclarecido. Alguns dos mecanismos propostos são: a inibição da síntese de

ácidos nucleicos, inibição das funções da membrana citoplasmática e inibição do metabolismo energético (285).

6. CONCLUSÕES

Os resultados obtidos nos trabalhos realizados no âmbito desta dissertação permitiram chegar às seguintes conclusões:

1. O perfil de compostos polifenólicos da couve tronchuda é constituído maioritariamente por heterósidos do campferol que podem estar acilados com ácidos hidroxicinâmicos e por heterósidos não flavonólicos de ácidos hidroxicinâmicos.

2. No perfil polifenólico dos extractos das sementes e das plântulas predominam os derivados dos ácidos hidroxicinâmicos; nas folhas internas existem derivados de ácidos hidroxicinâmicos e de flavonóis e os extractos de folhas externas, inflorescências e larvas são caracterizadas pela presença de derivados de flavonóis. O perfil polifenólico dos rebentos caulinares é caracterizado pela presença de vários compostos de cada uma das 3 sub-classes atrás referidas, isto é, derivados de ácidos hidroxicinâmicos, de flavonóis e de ácidos clorogénicos.

3. Globalmente, foram identificados 79 compostos polifenólicos diferentes: 51 heterósidos flavonólicos, 22 heterósidos de ácidos hidroxicinâmicos e 6 ácidos clorogénicos. Nos rebentos caulinares foram identificados 37 compostos polifenólicos diferentes, sendo esta a matriz em que se identificou o maior número de compostos.

4. Nos extractos de folha externa foram identificados, pela primeira vez na natureza, vários derivados acilados do campferol (campferol 3-*O*-(metoxicafeoil/cafeoil)-soforósido-7-*O*-glucósido, campferol 3-*O*-(sinapoil/cafeoil)-soforósido-7-*O*-glucósido, campferol 3-*O*- (feruloil/cafeoil)-soforóside-7-*O*-glucósido, campferol 3-*O*-(feruloil)-soforotriósido, campferol 3-*O*-(feruloil)-soforósido). Foi ainda identificado um composto contendo 6 glucoses, o campferol 3-*O*-tetraglucósido-7-*O*-soforósido.

5. De todas as amostras estudadas, os rebentos caulinares obtidos por micropropagação, utilizando o meio MSM líquido, suplementado com 2 mg/L de BAP e 0,1 mg/L de NAA, foram as que produziram a maior variedade de metabolitos polifenólicos.

6. Verificou-se que o perfil metabólico e a concentração dos compostos polifenólicos na couve tronchuda é afectada por muitos factores, incluindo o tipo de matriz, o tipo de produção (biológica ou convencional), a época de colheita, o clima e o processo extractivo.

7. Nos extractos aquosos de couve tronchuda, a quantidade de compostos polifenólicos podem atingir os 30 g/kg de peso seco de extracto (1,2 g/kg de peso fresco).

8. O perfil de ácidos orgânicos de todas as matrizes de couve tronchuda estudadas é muito semelhante. Os ácidos málico e cítrico são maioritários, representando cerca de 90% do teor de ácidos orgânicos. Os ácidos aconítico, xiquímico e fumárico são comuns a todas as matrizes estudadas.

9. O perfil de aminoácidos livres da couve tronchuda é característico. Na folha externa os aminoácidos maioritários são a prolina (34,1%) e a arginina (23,2%). Nas folhas internas os aminoácidos maioritários são a arginina (47,0%) e a treonina (12,7%). Os ácidos aspártico e glutámico e as suas amidas ácidas, que são habitualmente os aminoácidos maioritários nas outras plantas, representam cada um deles menos de 10% dos aminoácidos livres em todos os extractos analisados.

10. A concentração de aminoácidos livres nos extractos de couve tronchuda, atinge os 14,4 g/kg de peso fresco, uma concentração muito superior ao que habitualmente se encontra nas plantas (20-200 mg/kg de peso fresco).

11. No perfil de compostos voláteis dos extractos de couve tronchuda foram identificados 71 compostos, entre os quais 16 compostos de enxofre com origem provável na degradação dos glucosinolatos.

12. Os extractos de couve tronchuda demonstraram ter a capacidade de neutralizar várias espécies reactivas em sistemas químicos, incluindo o radical hidroxilo, o radical superóxido, o óxido nítrico e o peroxinitrito. A capacidade de captação do ácido hipocloroso foi pouco significativa.

13. As matrizes que demonstraram ter um maior potencial antioxidante foram a larva de *P. Brassicae* e os rebentos caulinares. A actividade antioxidante da larva de *P. brassicae* foi melhor do que a da couve que lhe serviu de alimento, e este resultado deu origem a uma patente para a obtenção de antioxidantes naturais a partir da larva.

14. As folhas internas e externas, as inflorescências e sobretudo as larvas de *P. brassicae* demonstraram ter capacidade de inibição da enzima xantina oxidase.

15. Os resultados do potencial antioxidante da couve tronchuda não foram confirmados num ensaio realizado com hepatócitos primários de rato submetidos a stress oxidativo intenso. Pelo contrário, os extractos em concentrações superiores a 800 µg/mL agravaram os efeitos dos hepatócitos expostos ao paraquato, usado para induzir stress oxidativo.

16. O extracto de couve tronchuda por si só não demonstrou ter potencial hepatotóxico *in vitro*, nas concentrações de 3,1 a 4000 µg/mL

PARTE IV

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GLOSSÁRIO

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METABOLOMA Colecção completa e quantificada de compostos de baixo peso molecular presentes numa célula, tecido ou organismo que participam em reacções metabólicas necessárias ao seu crescimento e funcionamento normal. Comparados com as 4 bases dos genes e os 20 aminoácidos das proteínas, os metabolitos são química e fisicamente mais variados, o que se deve às múltiplas variações nos arranjos atómicos. A complexidade dos metabolomas é ainda intensificada pela vasta gama de concentrações a ser detectadas (molar a sub-picomolar) (3, 4).

Os metabolitos podem ter uma origem endógena, ser sintetizados ou catabolizados bioquimicamente no organismo, ou podem derivar de compostos exógenos, como é o caso dos medicamentos e alimentos. A metabolómica, isto é, a identificação e quantificação imparcial de todos os metabolitos presentes num sistema biológico, não é exequível devido à sua enorme complexidade e tamanho. Por isso, o estudo do metaboloma é habitualmente compartimentado em perfil metabólico, assinatura metabólica, análise de metabolitos alvo, pegada metabólica e metabonómica (5).

PERFIL METABÓLICO Descrição de uma vasta gama de metabolitos, geralmente agrupados em classes, usando plataformas analíticas que permitam obter uma cobertura tão vasta quanto possível. Esta estratégia também pode ser descrita como perfil de metabolitos ou análise abrangente, e inclui, sempre que possível a identificação dos metabolitos. As variações relativas da resposta (relacionadas com a variação na concentração do metabolito) são utilizadas para definir as diferenças metabólicas. A descrição metabólica é geralmente aplicada numa estratégia indutora experimental em que os metabolitos com interesse biológico não são conhecidos *a priori*. O perfil metabólico exaustivo de plantas deve incluir hidratos de carbono, aminoácidos, ácidos orgânicos, lípidos/ácidos gordos e glucosinolatos, vitaminas e classes de metabolitos secundários como os fenilpropanóides, os terpenóides e os alcalóides, de acordo com a espécie em estudo (130).

ASSINATURA METABÓLICA Caracterização intensiva da amostra em bruto ou com pouca preparação num momento preciso. A identificação e quantificação são limitadas, sendo esta estratégia utilizada como uma ferramenta para descriminar entre amostras com diferentes origens biológicas ou estados. Usando as ferramentas adequadas, podem identificar-se potenciais biomarcadores, que rapidamente

permitem a distinção entre espécies. As assinaturas metabólicas podem ser sobrevalorizadas pois os sinais adequados para distinguir amostras nem sempre são biologicamente relevantes ou aplicáveis a outras situações (7). A assinatura metabólica também é definida como o estudo do metaboloma intracelular.

ANÁLISE DE METABOLITOS ALVO Identificação e quantificação completa de um só metabolito ou de um grupo de metabolitos estritamente relacionados, após uma preparação exaustiva da amostra para separar os metabolitos da matriz da amostra.

PEGADA METABÓLICA é a análise do metaboloma extracelular de um organismo, composto pelos metabolitos do meio não consumidos e dos metabolitos intracelulares excretados. Ao contrário dos metabolomas intracelulares não é necessário parar instantaneamente o metabolismo.

METABONÓMICA Medição multiparamétrica quantificada da resposta metabólica dinâmica dos sistemas vivos a estímulos patofisiológicos ou modificações genéticas.