**Faculdade de Farmácia**

**Universidade do Porto**

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## perfil em compostos fenólicos, ácidos orgânicos e aminoácidos livres e avaliação do potencial antioxidante

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*Aos meus Pais*

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2. Silva, B.M.; Andrade, P.B.; Mendes, G.C.; Seabra, R.M.; Ferreira, M.A. Study of the organic acids composition of quince (*Cydonia oblonga* Miller) fruit and jam. *J. Agric. Food Chem.* **2002**, *50*, 2313-2317.
3. Silva, B.M.; Andrade, P.B.; Ferreres, F.; Domingues, A.L.; Seabra, R.M.; Ferreira, M.A. Phenolic profile of quince fruit (*Cydonia oblonga* Miller) (pulp and peel). *J. Agric. Food Chem.* **2002**, *50*, 4615-4618.
4. Ferreres, F.; Silva, B.M.; Andrade, P.B.; Seabra, R.M.; Ferreira, M.A. Approach to the study of *C*-glycosyl flavones by Ion Trap HPLC-PAD-ESI/MS/MS: application to seeds of quince (*Cydonia oblonga*). *Phytochem. Anal.* **2003**, *14*, 352-359.
5. Silva, B.M.; Casal, S.; Andrade, P.B.; Seabra, R.M.; Oliveira, M.B.; Ferreira, M.A. Development and evaluation of a GC/FID method for the analysis of free amino acids in quince fruit and jam. *Analyt. Sci.* **2003**, *19*, 1285-1290.
6. Silva, B.M.; Casal, S.; Andrade, P.B.; Seabra, R.M.; Oliveira, M.B.; Ferreira, M.A. Free amino acid composition of quince (*Cydonia oblonga* Miller) fruit (pulp and peel) and jam. *J. Agric. Food Chem.* **2004**, *52*, 1201-1206.
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amino acids in quince fruit (*Cydonia oblonga* Miller). *Eur. Food Res. Technol.* **2004**, *218*, 385-389.

9. Silva, B.M.; Andrade, P.B.; Martins, R.C.; Valentão, P.; Ferreres, F.; Seabra, R.M.; Ferreira, M.A. Quince (*Cydonia oblonga* Miller) fruit characterization using Principal Component Analysis. *J. Agric. Food Chem.* **2005**, *53*, 111-122.
10. Silva, B.M.; Andrade, P.B.; Ferreres, F.; Seabra, R.M.; Oliveira, M.B.; Ferreira, M.A. Composition of quince (*Cydonia oblonga* Miller) seeds: phenolics, organic acids and free amino acids. *Nat. Prod. Res.* **2005**, *19*, 275-281.
11. Silva, B.M.; Andrade, P.B.; Martins, R.C.; Seabra, R.M., Ferreira, M.A. Principal Component Analysis as tool of characterization of quince (*Cydonia oblonga* Miller) jam. *Food Chem.* (*in press*).
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*Capítulos de livros:*

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6. Silva, B.M.; Ferreres, F.; Andrade, P.B.; Gonçalves, A.C.; Seabra, R.M.; Ferreira, M.A. C-glycosyl flavones from *Cydonia oblonga* seeds. Painel. *1st International Conference on Polyphenols and Health*. 18 a 21 de Novembro de 2003. Vichy (France).
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# ABREVIATURAS E SÍMBOLOS

| | |
|---|---|
| Ala | alanina |
| ANOVA | Analysis of variance |
| APCI | Atmospheric Pressure Chemical Ionization |
| Asn | asparagina |
| Asp | ácido aspártico |
| CC | Cromatografia em Coluna |
| $CDCl_3$ | clorofórmio deuterado |
| CID | Collision-Induced Dissociation |
| $\lambda_{máx}$ | comprimento de onda máximo em nm |
| COSY | Correlated Spectroscopy |
| 3-CQA | ácido 3-*O*-cafeoilquínico |
| 4-CQA | ácido 4-*O*-cafeoilquínico |
| 5-CQA | ácido 5-*O*-cafeoilquínico |
| 3,5-diCQA | ácido 3,5-*O*-dicafeoilquínico |
| Cys | cisteína |
| CV | coeficiente de variação |
| $\delta$ | desvio químico em ppm |
| DAD | Diode Array Detector |
| DCI | Desorption Chemical Ionization |
| DEPT | Distortionless Enhancement by Polarization Transfer |
| DMSO-*d6* | dimetilsulfóxido hexadeuterado |
| DPPH | 1,1'-difenil-2-picrilhidrazilo |
| EC | End-Capped |
| ECF | cloroformato de etilo |
| EI | Electron Impact |
| ESI | ElectroSpray Ionization |
| FAB | Fast Atom Bombardment |
| FID | Flame Ionization Detector |
| GC | Gas Chromatography |
| Gln | glutamina |
| Gly | glicina |

| | |
|---|---|
| Glu | ácido glutâmico |
| His | histidina |
| HMBC | Heteronuclear Multiple Bond Correlation |
| HMF | 5-hidroximetilfurfuraldeído |
| HPLC | High Performance Liquid Chromatography |
| HSQC | Heteronuclear Single Quantum Correlation |
| Hyp | hidroxiprolina |
| $IC_{50}$ | concentração inibitória de 50% da reacção |
| Ile | isoleucina |
| INE | Instituto Nacional de Estatística |
| IS | internal standard |
| *J* | constante de acoplamento |
| K-3-Gly | glicósido do campferol |
| K-Gly-pC1 e K-Gly-pC2 | glicósidos do campferol acilados com ácido *p*-cumárico |
| K-3-Glu | 3-*O*-glucosilcampferol |
| K-3-Rut | 3-*O*-rutinosilcampferol |
| Leu | leucina |
| Lys | lisina |
| MeOH | metanol |
| Met | metionina |
| $[M-H]^-$ | ião pseudomolecular |
| MIKE | Mass analysed Ion Kinetic Energy |
| MS | Mass Spectrometry |
| *m/z* | relação massa/carga dos fragmentos iónicos formados num espectro de massa |
| nd | não detectado |
| NEC | Non End-Capped |
| NMR | Nuclear Magnetic Resonance |
| nq | não quantificado |
| Orn | ornitina |
| *p* | probabilidade |

| | |
|---|---|
| PAD | Photodiode Array Detection |
| PC | Principal Component |
| PCA | Principal Component Analysis |
| Phe | fenilalanina |
| Pro | prolina |
| Q-3-Gal | 3-*O*-galactosilquercetina |
| Q-Gly-pC1 e Q-Gly-pC2 | glicósidos da quercetina acilados com ácido *p*-cumárico |
| Q-3-Rut | rutina |
| R | coeficiente de correlação |
| RT | retention time |
| Σ | somatório |
| SCX | Strong Cation Exchange |
| SD | standard deviation |
| Ser | serina |
| SPE | Solid-Phase Extraction |
| Thr | treonina |
| tr | traces |
| Trp | triptofano |
| Tyr | tirosina |
| UV | Ultra-Violeta |
| Val | valina |
| Vis | Visível |

NOTA: para algumas abreviaturas foi mantida a nomenclatura anglo-saxónica dado o seu carácter universal, facilitando assim o seu reconhecimento.

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# Índice Geral

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**RESUMO**

# Resumo

Nesta dissertação de doutoramento foi estudada a composição química do marmelo e da marmelada, em termos de compostos fenólicos, ácidos orgânicos e aminoácidos livres.

Numa primeira etapa, desenvolveram-se os métodos analíticos cromatográficos com detecções diversas necessários à quantificação dos diferentes compostos para, numa segunda fase, os aplicar ao estudo das diversas amostras. De seguida, aplicou-se a PCA aos resultados obtidos.

As amostras de marmelo, as quais foram subdivididas em polpa (mesocarpo), casca (epicarpo) e semente, provieram de nove regiões de Portugal (Amarante, Baião, Bragança, Caminha, Covilhã, Custóias, Pinhel, Vila Real e Viseu) e foram colhidas em três anos consecutivos (2000-2002), num total de cinquenta e uma amostras.

As amostras de marmelada incluíram vinte marcas comerciais (produzidas de modo tradicional e industrial), as quais foram comercializadas em três anos consecutivos de fabricação (2000-2002), e ainda duas preparadas de modo caseiro, num total de cinquenta e uma amostras.

Os compostos fenólicos foram analisados por HPLC/DAD, após extracção com água ácida, seguida de purificação em colunas de extracção em fase sólida ISOLUTE C18 (NEC). Para a identificação dos flavonóides das cascas e das sementes de marmelo e das marmeladas, recorreu-se a uma técnica de Ion Trap HPLC-DAD-ESI/MS/MS.

O perfil fenólico das três partes do marmelo mostrou-se distinto. As polpas apresentaram seis compostos fenólicos: os ácidos 3-*O*-, 4-*O*- e 5-*O*-cafeoilquínicos e 3,5-*O*-dicafeoilquínico, a 3-*O*-galactosilquercetina e a rutina. As cascas apresentaram treze compostos fenólicos: os seis presentes nas polpas e ainda o 3-*O*-glucosilcampferol, o 3-*O*-rutinosilcampferol e cinco compostos parcialmente identificados (um glicósido do campferol, dois glicósidos da quercetina acilados com ácido *p*-cumárico e dois glicósidos do campferol também acilados com ácido *p*-cumárico). As sementes apresentaram um perfil fenólico característico, composto pelos mesmos ácidos cafeoilquínicos e por diversas *C*-glicosil flavonas: lucenina-2, vicenina-2, estelarina-2, isoschaftósido, schaftósido, 6-*C*-pentosil-8-*C*-glucosil crisoeriol e 6-*C*-glucosil-8-*C*-pentosil crisoeriol.

Todas as amostras de marmelada apresentaram um perfil composto por, pelo menos, os seis compostos fenólicos presentes nas polpas. No entanto, algumas amostras, sobretudo as

produzidas industrialmente, também continham os compostos característicos das cascas. A presença destes compostos indica assim uma adulteração das marmeladas por utilização de frutos com casca.

No decurso do estudo da composição fenólica do marmelo (polpa e casca) e da marmelada, foi isolado e identificado um composto terpénico, o β-D-glucopiranósido do ácido 9-amino-2,7-dimetil-8-hidroxinona-2,4-dienóico. Para esse efeito recorreu-se a métodos cromatográficos (CC e HPLC semi-preparativa), espectrofotométricos (UV) e espectrométricos ($^{13}C$ e $^{1}H$ NMR e MS). Também foram detectados dois compostos com espectro UV idêntico. Posteriormente, foi realizada a respectiva quantificação dos três. Estes compostos terpénicos foram descritos pela primeira vez na natureza, podendo ser considerados potenciais marcadores químicos deste fruto e dos seus derivados.

No que diz respeito à metodologia desenvolvida para a identificação e quantificação de ácidos orgânicos por HPLC/UV, verificou-se que a preparação da amostra é simples, envolvendo extracção com metanol (40ºC) e filtração através de colunas de extracção em fase sólida ISOLUTE C18 (NEC).

O marmelo (polpa e casca) e a marmelada apresentaram um perfil em ácidos orgânicos idêntico, sendo geralmente composto por sete compostos: os ácidos oxálico, cítrico, ascórbico, málico, quínico, shiquímico e fumárico. No que diz respeito ao perfil em ácidos orgânicos das sementes, verificou-se a presença apenas de seis dos sete ácidos presentes nas polpas e nas cascas (o ácido orgânico ausente foi o ácido oxálico).

As polpas e as cascas evidenciaram quantidades elevadas de ácidos málico e quínico, sendo a sua soma sempre superior a 90% do total dos ácidos orgânicos. No entanto, nas sementes o valor médio da soma destes dois ácidos foi de 55% e os ácidos cítrico e ascórbico apresentaram-se em percentagens apreciáveis, de cerca de 24 e 20%, respectivamente.

Tal como as polpas e as cascas, as marmeladas apresentaram igualmente elevados teores de ácidos málico e quínico, embora o seu valor médio tivesse sido um pouco inferior ao do marmelo. As quantidades apreciáveis de ácidos cítrico e ascórbico encontradas podem ser reveladoras do uso destes produtos como aditivos reguladores de acidez e antioxidantes.

Relativamente à determinação de aminoácidos livres, foi desenvolvida uma metodologia de GC/FID, em que a preparação da amostra é simples, envolvendo a purificação dos extractos

em coluna de extracção em fase sólida SCX e um processo de derivatização com cloroformato de etilo.

Todas as amostras apresentaram um perfil em aminoácidos livres qualitativo idêntico, com vinte e um aminoácidos. Verificou-se uma predominância de dois aminoácidos livres: a asparagina e o ácido aspártico. A polpa é caracterizada por teores superiores de hidroxiprolina e inferiores de ácido glutâmico comparativamente aos existentes na casca.

Aos dados obtidos aplicou-se a PCA de forma a avaliar a influência de três factores na composição do marmelo, em termos de compostos fenólicos, ácidos orgânicos e aminoácidos livres – a parte do fruto, a sua origem geográfica e o seu ano de colheita. Verificou-se que a determinação do perfil fenólico é a mais interessante, sobretudo no que diz respeito à discriminação das diferentes partes do fruto. Nesta análise verificou-se que duas PCs descrevem 81,29% da variabilidade total, PC1 (74,14%) e PC2 (7,15%). A PC1 descreve as diferenças entre os conteúdos em ácidos cafeoilquínicos e em flavonóides, o que caracteriza a diferença entre a polpa e a casca; enquanto que a PC2 relaciona o conteúdo de ácido 4-*O*-cafeoilquínico e de ácidos 5-*O*-cafeoilquínico e 3,5-*O*-dicafeoilquínico. Assim, a PCA do perfil fenólico permite a discriminação clara entre polpa e casca de marmelo. A PCA dos perfis em ácidos orgânicos e dos aminoácidos livres não permite a referida distinção. Não se encontrou correlação directa entre os perfis em compostos fenólicos, ácidos orgânicos e aminoácidos livres.

Também se realizou a PCA de todos os dados obtidos. Nesta análise verificou-se que três PCs explicam 50,86% da variabilidade total: PC1 (24,60%), PC2 (16,78%) e PC3 (9,49%). A PC1 enfatiza as diferenças na composição fenólica da polpa e da casca; a PC2 apresenta as diferenças entre os conteúdos em ácidos cafeoilquínicos e flavonóides da polpa e da casca, bem como pequenas diferenças na composição em ácidos orgânicos e aminoácidos livres. A PC3 descreve a variação em termos de ácidos orgânicos e a ortogonalidade existente entre os ácidos 3,5-*O*-dicafeoílquinico e 4-*O*-cafeoilquínico.

Também foi aplicada a PCA aos dados relativos às marmeladas de forma a avaliar a influência de três factores na sua composição – a marca comercial, o modo de preparação e o ano de comercialização. Verificou-se, mais uma vez, que a determinação do perfil fenólico é a mais interessante. A PCA enfatiza as diferenças entre as marmeladas preparadas de modo tradicional e industrial. Nesta análise duas PCs descrevem 54,38% da variabilidade total, PC1 (37,41%) e PC2 (16,97%). A PC1 descreve as diferenças entre os conteúdos em ácidos 3-*O*- e 5-*O*-

cafeoilquínicos e em todos os flavonóides; enquanto que a PC2 relaciona os conteúdos de ácidos 4-*O*- e 5-*O*-cafeoilquínico e de ácidos 3-*O*-cafeoilquínico e 3,5-*O*-dicafeoilquínico. Verificou-se que, de uma maneira geral, os industriais usam marmelos por descascar na preparação das marmeladas, o que, de acordo com a Legislação Portuguesa, constitui uma adulteração. A PCA dos perfis em ácidos orgânicos e dos aminoácidos livres não permite a distinção entre marmeladas produzidas de modo tradicional e industrial. Neste caso, também não se encontrou correlação directa entre os perfis em compostos fenólicos, ácidos orgânicos e aminoácidos livres.

Embora o processamento térmico do marmelo durante a produção da marmelada provoque a isomerização dos ácidos cafeoilquínicos e leve à degradação de alguns ácidos orgânicos (ácidos cítrico, ascórbico e shiquímico), os conteúdos totais dos compostos fenólicos e dos ácidos orgânicos determinados não sofreram alterações apreciáveis. Contudo, o perfil em aminoácidos livres alterou-se durante o referido processamento, provavelmente devido à hidrólise de proteínas, peptídeos e outras moléculas com aminoácidos na sua constituição.

Por último, efectuou-se uma comparação da actividade antirradicalar dos extractos metanólicos completos com as suas duas fracções, a fenólica e a dos ácidos orgânicos. A actividade antirradicalar dos extractos foi avaliada através de um microensaio com o radical DPPH. A fracção fenólica apresentou sempre actividade superior à do extracto metanólico completo, enquanto que a de ácidos orgânicos teve a actividade mais reduzida dos três tipos, o que parece indicar que a fracção fenólica é a que fornece a maior contribuição para o potencial antioxidante de marmelos e marmeladas.

De entre os extractos metanólicos completos, o de casca apresentou uma capacidade antioxidante superior (valores de $IC_{50}$ de 1,7, 0,6, 2,0, 8,9 e 8,4 mg/ml para extractos metanólicos de polpa, casca, semente, marmelada preparada com polpa de marmelo e marmelada preparada com marmelos por descascar, respectivamente). Os valores de $IC_{50}$ dos extractos metanólicos de polpa, casca e marmeladas correlacionaram-se com o conteúdo total em ácidos cafeoilquínicos.

No que diz respeito às fracções fenólicas, a de semente apresentou uma actividade antioxidante mais marcada (valores de $IC_{50}$ de 1,0, 0,4, 0,1, 7,0 e 6,0 mg/ml para fracções fenólicas de polpa, casca, semente, marmelada preparada com polpa de marmelo e marmelada preparada com marmelos por descascar, respectivamente). Os valores de $IC_{50}$ das fracções fenólicas de polpa, casca e marmeladas estiveram fortemente correlacionados com os conteúdos totais em ácidos cafeoilquínicos e em fenóis.

Uma actividade antirradicalar mais forte foi observada na fracção de ácidos orgânicos da casca (valores de $IC_{50}$ de 11,6, 6,9, 12,9, 22,6 e 16,3 mg/ml para fracções de ácidos orgânicos de polpa, casca, semente, marmelada preparada com polpa de marmelo e marmelada preparada com marmelos por descascar, respectivamente). Os valores de $IC_{50}$ destas fracções de polpa, casca e marmeladas correlacionaram-se com os conteúdos em ácidos ascórbico e cítrico.

**ABSTRACT**

## Abstract

In this dissertation the chemical composition of quince fruit and jam was studied, in terms of phenolic compounds, organic acids and free amino acids.

First, the analytical chromatographic methods with several detections were developed and validated; second, these methods were applied to the study of the several samples. Then, PCA was applied to the obtained data.

Quince fruit samples, which were divided in pulp (mesocarp), peel (epicarp) and seed, were collected in nine geographical origins of Portugal (Amarante, Baião, Bragança, Caminha, Covilhã, Custóias, Pinhel, Vila Real and Viseu), harvested in three consecutive years (2000-2002), in a total of fifty one samples.

Quince jam samples included twenty different brands (traditionally and industrially prepared), commercialised in three consecutive years of production (2000-2002), and two homemade samples, in a total of fifty one samples.

Phenolic compounds were analysed by HPLC/DAD, after extraction with acid water and purification, by using solid-phase extraction columns ISOLUTE C18 (NEC). An Ion Trap HPLC-DAD-ESI/MS/MS technique was necessary for the identification of quince peel and seed and jam flavonoids.

The phenolic profile of the three parts of quince fruit was distinct. The pulps presented six phenolic compounds: 3-*O*-, 4-*O*- and 5-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids, 3-*O*-galactosylquercetin and rutin. The peels presented thirteen phenolic compounds: the same that were found in pulps, plus 3-*O*-glucosylkaempferol, 3-*O*-rutinosylkaempferol and five partially identified compounds (one kaempferol glycoside, two quercetin glycosides acylated with *p*-coumaric acid and two kaempferol glycosides also acylated with *p*-coumaric acid). The seeds presented a characteristic phenolic profile, composed by the same caffeoylquinic acids and by several *C*-glicosyl flavones: lucenin-2, vicenin-2, stellarin-2, isoschaftoside, schaftoside, 6-*C*-pentosyl-8-*C*-glucosyl chrysoeriol e 6-*C*-glucosyl-8-*C*-pentosyl chrysoeriol.

All the quince jam samples presented a phenolic profile composed by, at least, the six compounds present in the pulps. However, some samples, especially the industrially prepared ones, also contained the characteristic compounds of peels. The presence of these compounds indicates quince jam adulteration by use of unpeeled fruits.

In the course of the phenolic composition study of quince fruit (pulp and peel) and jam, a terpenic compound was isolated and identified, the β-D-glucopyranoside of 9-amino-2,7-dimethyl-8-hydroxynona-2,4-dienoic acid. For this purpose, chromatographic (CC and semi-preparative HPLC) and spectroscopic (UV, $^{13}C$ and $^{1}H$ NMR, and MS) means were used. Other two chemically related compounds, with similar UV spectra, were also detected. Subsequently, these three compounds were also quantified. These terpenic compounds were described for the first time in nature and can be used as a tool for the characterization of quince and its derivatives.

In what concerns the developed and validated methodology for organic acids identification and quantification by HPLC/UV, the sample preparation is simple, involving methanolic extraction (40°C) and filtration throughout solid-phase extraction columns ISOLUTE C18 (NEC).

Quince fruit (pulp and peel) and jam presented an identical organic acids profile, generally composed by seven compounds: oxalic, citric, ascorbic, malic, quinic, shikimic and fumaric acids. Seeds organic acids profile was composed by six of the seven acids presented in pulps and peels (the absent one was oxalic acid).

Pulps and peels were characterized by large amounts of malic and quinic acids, being their sum always higher than 90% of all the determined organic acids. Nevertheless, seeds presented a mean value of the referred sum of 55%, and citric and ascorbic acids were present in considerable amounts of approximately 24 and 20%, respectively.

As pulps and peels, quince jams presented high malic plus quinic acids content; nevertheless, the mean value was lower. The considerable amounts of citric and ascorbic acids found may reveal the use of these products as acidity regulators and antioxidants.

Concerning the free amino acids determination, a GC/FID methodology was developed and validated. The sample preparation is simple, involving an extract purification step by using SCX solid-phase extraction column and a derivatization procedure with ethyl chloroformate.

All samples presented an identical qualitative free amino acids profile, composed by twenty-one amino acids. The two most abundant amino acids were asparagin and aspartic acid. Pulp is characterized by higher hydroxyproline and lower glutamic acid contents than peel.

PCA was performed in order to evaluate the influence of three factors in the quince fruit chemical composition, in terms of phenolics, organic acids and free amino acids - quince fruit part, geographical origin and harvesting year. Phenolics determination is the most interesting, in

what concerns the discrimination of the different parts of the fruit. Two PCs accounted 81.29% of the total variability, PC1 (74.14%) and PC2 (7.15%). The PC1 describes the differences between the contents of caffeoylquinic acids and flavonoids, which characterizes the difference between quince pulp and peel; while PC2 relates the content of 4-*O*-caffeoylquinic acid and the contents of 5-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids. PCA of phenolic compounds enable a clear distinction between the two parts of the fruit. PCA of organic acids and free amino acids does not allow the referred distinction. There was no direct correlation between the phenolic, organic acids and free amino acids profiles.

PCA was also applied to the global data. Three PCs explain 50.86% of the variability of all data: PC1 (24.60%), PC2 (16.78%) and PC3 (9.49%). PC1 emphasizes the differences in terms of phenolic composition between pulp and peel; PC2 present the differences between caffeoylquinic acids and flavonoids contents of pulp and peel, as well as the small differences in organic acid and free amino acid composition; PC3 describes the variation in terms of organic acids and the orthogonality existent between 3,5-*O*-dicaffeoylquinic acid and 4-*O*-caffeoylquinic acid.

PCA was also performed in order to evaluate the influence of three factors in quince jam composition – brand, preparation type and commercialization year. Once more, the phenolics determination is the most interesting. The differences between traditional and industrial quince jams phenolic profile were emphasised during PCA. Two PCs described 54.38% of all variance, PC1 (37.41%) and PC2 (16.97%). The PC1 describes the differences between the contents of 3-*O*- and 5-*O*-caffeoylquinic acids and all flavonoids; while PC2 relates the contents of 4-*O*- and 5-*O*-caffeoylquinic acids and 3-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids. It seems that many industrial manufacturers usually use unpeeled fruits in the preparation of the jams, which, according to the Portuguese Legislation, constitutes an adulteration. PCA of organic acids and free amino acids does not allow the distinction between traditionally and industrially prepared quince jams and there was no direct correlation between the phenolic, organic acids and free amino acids profiles.

Although quince fruit thermal processing used during jam production leads to caffeoylquinic acids isomerisation and to the degradation of some organic acids (citric, ascorbic and shikimic acids), the total contents of the determined phenolics and organic acids in quince fruit and jam did not indicated appreciable changes. Nevertheless, free amino acids profile was

changed by the referred processing, probably due to hydrolysis of proteins, peptides and other amino acids-derived compounds.

Finally, a comparison of the antiradical activity between complete methanolic extracts and their two fractions, phenolic and organic acid ones, was performed. The extracts antiradical activity was evaluated by using a micro assay with DPPH radical. Phenolic fraction exhibited always a stronger antioxidant activity than the whole methanolic extract, while organic acid fraction was always the weakest in terms of antiradical activity, which seems to indicate that the phenolic fraction gives the major contribution for the antioxidant potential of quince fruit and jam.

The evaluation of the antioxidant activity of methanolic extracts showed that peel extract was the one presenting the highest antioxidant capacity ($IC_{50}$ values of 1.7, 0.6, 2.0, 8.9 and 8.4 mg/ml for methanolic extracts of quince pulp, peel, seed and jams, prepared with peeled and unpeeled fruits, respectively). $IC_{50}$ of quince pulp, peel and jams extracts were correlated with the caffeoylquinic acids total content.

Among the phenolic fractions, seed extract was the one that exhibited the strongest antioxidant activity ($IC_{50}$ values of 1.0, 0.4, 0.1, 7.0 and 6.0 mg/ml for phenolic fractions of quince pulp, peel, seed and jams, prepared with peeled and unpeeled fruits, respectively). $IC_{50}$ of quince pulp, peel and jams phenolic extracts were strongly correlated with caffeoylquinic acids and phenolics total contents.

For organic acids fractions, peel extract was the one with the strongest antiradical activity ($IC_{50}$ values of 11.6, 6.9, 12.9, 22.6 and 16.3 mg/ml for organic acid fractions of quince pulp, peel, seed and jams, prepared with peeled and unpeeled fruits, respectively). The $IC_{50}$ values of quince pulp, peel and jams organic acid fractions were correlated with the ascorbic acid and citric acid contents.

**RÉSUMÉ**

# Résumé

Dans cette dissertation a été étudié la composition chimique du coing et de la marmelade, en ce qui concerne de composés phénoliques, acides organiques et acides aminés libres.

Dans une première étape, on a développée les méthodes analytiques chromatographiques avec divers détections nécessaires à la quantification des différents composées pour, dans une seconde phase, les appliquer à l'étude des divers échantillons. De suivante, on a appliquée PCA aux résultats obtenus.

Les échantillons de coing, qui ont été divisées dans pulpe (mésocarpe), peau (épicarpe) et graine, sont venus de neuf régions du Portugal (Amarante, Baião, Bragança, Caminha, Covilhã, Custóias, Pinhel, Vila Real et Viseu) et ont été récoltés dans trois ans consécutifs (2000-2002), dans un total des cinquante et un échantillons.

Les échantillons de marmelade ont inclus vingt marques commerciales (produites de manière traditionnelle et industrielle), qui ont été commercialisées dans les mêmes trois ans consécutifs de fabrication (2000-2002), et encore deux préparées de manière gardien, dans un total des cinquante et un échantillons.

Les composés phénoliques ont été analysés par HPLC/DAD, après extraction avec eau acide, suivante de purification en colonnes d'extraction dans phase solide ISOLUTE C18 (NEC). Pour l'identification de flavonoïdes des peaux et des graines de coing et des marmelades, s'a fait appel à une technique d'Ion Trap HPLC-DAD-ESI/MS/MS.

Le profil phénolique des trois parties du coing s'est montré distinct. Les pulpes ont présenté six composés phénoliques: les acides 3-*O*-, 4-*O*- et 5-*O*-caféylquiniques et 3,5-*O*-dicaféylquinique, 3-*O*-galactosylquércetine et rutine. Les peaux ont présenté treize composés phénoliques: les six présents dans les pulpes et encore 3-*O*-glucosylkaempférol, 3-*O*-rutinosylkaempférol et cinq composés partiellement identifiés (une glycoside de kaempférol, deux glycosides de quércetine acylés avec acide *p*-coumarique et deux glycosides de kaempférol aussi acylés avec acide *p*-coumarique). Les graines ont présenté un profil phénolique caractéristique, composé des mêmes acides caféylquiniques et diverses *C*-glycosylflavones: lucénine-2, vicénine-2, stéllarine-2, isoshaftoside, shaftoside, 6-*C*-pentosyl-8-*C*-glucosyl chrysoeriol et 6-*C*-glucosyl-8-*C*-pentosyl chrysoeriol.

Toutes les échantillons de marmelade ont présenté un profil composé par, au moins, les six composés phénoliques présents dans les pulpes. Cependant, quelques échantillons, surtout produites industriellement, contenaient aussi les composés caractéristiques des peaux. La présence de ces composés indique ainsi une adultération des marmelades par utilisation de fruits avec de peau.

Au cours de l'étude de la composition phénolique du coing (pulpe et peau) et de la marmelade, un composé terpénique a été isolé et identifié, le β-D-glucopyranoside de l'acide 9-amino-2,7-diméthyl-8-hydroxynona-2,4-dienoique. À cet effet s'est fait appel à des méthodes chromatographiques (CC et HPLC semi-préparatif) et spectroscopiques (UV, $^{13}C$ et $^{1}H$ NMR et MS). Deux composés avec spectre d'UV identique ont été détectés aussi. Ultérieurement, on a été réalisé la quantification des trois. Ces composés terpéniques ont été décrits pour la première fois dans la nature, et pouvant être considérés des potentiels marqueurs chimiques de ce fruit et de leurs dérivés.

En ce qui concerne la méthodologie développée pour l'identification et la quantification d'acides organiques par HPLC/UV, s'est vérifié que la préparation de l'échantillon est simple, impliquant extraction avec méthanol (40°C) et filtration à travers des colonnes d'extraction dans phase solide ISOLUTE C18 (NEC).

Le coing (pulpe et peau) et la marmelade ont présenté un profil d'acides organiques identique, étant composés généralement de sept composés: les acides oxalique, citrique, ascorbique, malique, quinique, shikimique et fumarique. En ce qui concerne le profil d´acides organiques des graines, s'est vérifiée seulement la présence de six des sept acides présents dans les pulpes et dans les peaux (l'acide organique absente a été l'acide oxalique).

Les pulpes et les peaux ont présenté quantités élevées d'acides malique et quinique, étant leur addition toujours supérieure à 90% de tous les acides organiques. Cependant, dans les graines la valeur moyenne de l'addition de ceux deux acides a été de 55% et les acides citrique et ascorbique se sont présentées en pourcentages appréciables, d'environ 24 et 20%, respectivement.

Tel comme les pulpes et les peaux, les marmelades ont présenté également teneurs élevées d'acides malique et quinique, bien que sa valeur moyenne avait été un peu inférieur à ce du coing. Les quantités appréciables d'acides citrique et ascorbique trouvées peuvent être révélatrices de l'utilisation de ces produits comme additifs régulateurs d'acidité et antioxydants.

Relativement à la détermination d'acides aminés libres, on a été développé une méthodologie de GC/FID, dans que la préparation de l'échantillon est simple, impliquant une étape de purification des extraits en colonne de extraction dans phase solide SCX et un procédé de derivatization avec chloroformate éthylique.

Tous les échantillons ont présenté un profil qualitatif en acides aminés libres identique, avec vingt et un acides aminés. S'est vérifiée une prédominance de deux acides aminés libres: l'asparagine et l'acide aspartique. La pulpe est caractérisée par des teneurs supérieures de hydroxyproline et inférieurs d'acide glutamique aux existantes dans la peau.

Aux résultats obtenus s'est appliquée PCA de manière à évaluer l'influence de trois facteurs dans la composition du coing, en termes de composés phénoliques, d'acides organiques et d'acides aminés libres - la partie du fruit, son origine géographique et son année de récolte. Il s'est vérifié que la détermination du profil phénolique est la plus intéressante, surtout en ce qui concerne la discrimination des différentes parties du fruit. Dans cette analyse s'est vérifié que deux PCs décrivent 81.29% de la variabilité totale, PC1 (74.14%) et PC2 (7.15%). La PC1 décrit les différences entre les contenus des acides caféylquiniques et des flavonoïdes, ce que caractérise la différence entre la pulpe et la peau; tandis que la PC2 rapporte le contenu d'acide 4-*O*-caféylquinique et d'acides 5-*O*-caféylquinique et 3,5-*O*-dicaféylquinique. Ainsi, PCA du profil phénolique permet la discrimination claire entre pulpe et peau de coing. PCA des profils des acides organiques et des acides aminés libres ne permet pas cette distinction. Ne s'est pas trouvée corrélation directe entre les profils des composés phénoliques, d'acides organiques et d'acides aminés libres.

La PCA des toutes les résultats obtenus a été réalisé aussi. Dans cette analyse s'est vérifié que trois PCs expliquent 50.86% de la variabilité totale: PC1 (24.60%), PC2 (16.78%) et PC3 (9.49%). La PC1 souligne les différences dans la composition phénolique de la pulpe et de la peau; la PC2 présente les différences entre les contenus des acides caféylquiniques et des flavonoïdes de la pulpe et de la peau, ainsi que petites différences dans la composition d'acides organiques et d'acides aminés libres. La PC3 décrit la variation en termes d'acides organiques et l'orthogonalité existant entre les acides 3,5-*O*-dicaféylquinique et 4-*O*-caféylquinique.

La PCA a été appliquée aussi aux résultats relatifs aux marmelades de manière à évaluer l'influence de trois facteurs dans sa composition - la marque commerciale, la manière de préparation et l'année de commercialisation. Il s'est vérifié, encore une fois, que la détermination

du profil phénolique est la plus intéressante. PCA souligne les différences entre les marmelades préparées de manière traditionnelle et industrielle. Dans cette analyse deux PCs ont décrit 54.38% de la variabilité totale, PC1 (37.41%) et PC2 (16.97%). La PC1 décrit les différences entre les contenus des acides 3-*O*- et 5-*O*-caféylquiniques et tout les flavonoïdes; tandis que la PC2 rapporte les contenus d'acides 4-*O*- et 5-*O*-caféylquinique et d'acides 3-*O*-caféylquinique et 3,5-*O*-dicaféylquinique. S'est vérifié que, d'une manière générale, les industriels utilisent des coings non épluchés dans la préparation des marmelades, ce qui, conformément à la Législation Portugaise, constitue une adultération. PCA des profils des acides organiques et des acides aminés libres ne permet pas la distinction entre des marmelades produites de manière traditionnelle et industrielle. Dans ce cas, ne s'est pas trouvée aussi corrélation directe entre les profils des composés phénoliques, acides organiques et acides aminés libres.

Bien que le traitement thermique du coing pendant la production de la marmelade provoque la isomérisation des acides caféylquiniques et la dégradation de quelques acides organiques (acides citrique, ascorbique et shikimique), les contenus totaux des composés phénoliques et des acides organiques déterminés n'ont pas souffert modifications appréciables. Cependant, le profil d'acides aminés libres s'est modifié pendant le mentionné traitement, probablement dû à l'hydrolyse des protéines, des peptides et d'autres molécules avec des acides aminés dans sa constitution.

Finalement, s'est effectuée une comparaison de l'activité antiradicalar des extraits méthanoliques complètes avec leurs deux fractions, la phénolique et ce des acides organiques. L'activité antiradicalar des extraits a été évaluée à travers d'un micro essai avec le radical DPPH. La fraction phénolique a présenté toujours activité supérieur à la de l'extrait méthanolique complet, tandis que ce d'acides organiques a eu l'activité le plus bas des trois types, ce qui semble indiquer que la fraction phénolique donne une contribution plus élevée pour le potentiel antioxydant de coings et marmelades.

Entre les extraits de méthanoliques complètes, ce de peau a présenté la plus grande capacité antioxydant (valeurs de $IC_{50}$ de 1.7, 0.6, 2.0, 8.9 et 8.4 mg/ml pour des extraits méthanoliques de pulpe, peau, grain, marmelade préparée avec pulpe de coings et marmelade préparée avec coings deépluché, respectivement). Les valeurs d'$IC_{50}$ des extraits méthanoliques de pulpe, peau et marmelade ont été corrélées avec le contenu total d'acides caféylquiniques.

En ce qui concerne les fractions phénoliques, ce de graine a présenté une activité antioxydant plus marquée (valeurs de $IC_{50}$ de 1.0, 0.4, 0.1, 7.0 et 6.0 mg/ml pour fractions phénoliques de pulpe, peau, grain, marmelade préparée avec pulpe de coings et marmelade préparée avec coings deépluché, respectivement). Les valeurs d'$IC_{50}$ des fractions phénoliques de pulpe, peau et marmelade ont été fortement corrélées avec les contenus totaux d'acides caféylquiniques et de phénols.

Une activité antiradicalar plus forte a été observée avec la fraction d'acides organiques de la peau (valeurs de $IC_{50}$ de 11.6, 6.9, 12.9, 22.6 et 16.3 mg/ml pour fractions d'acides organiques de pulpe, peau, grain, marmelade préparée avec pulpe de coings et marmelade préparée avec coings deépluché, respectivement). Les valeurs d'$IC_{50}$ de ces fractions de pulpe, de peau et de marmelade ont été corrélées avec les contenus dans des acides ascorbique et citrique.

# ENQUADRAMENTO GERAL DA TESE E OBJECTIVOS

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## Enquadramento Geral da Tese

Nos últimos anos tornou-se evidente que existem riscos e benefícios relacionados com a saúde associados à dieta (Wildman, 2001). Os estudos nutricionais recomendam o consumo regular de frutos e vegetais, os quais constituem uma parte essencial da dieta Mediterrânica, de forma a favorecer uma boa qualidade de vida. Os frutos e os vegetais são óptimas fontes de vitaminas, especialmente de vitaminas A e C, excelentes fontes de fibra e, sendo naturalmente hipolipídicos, apresentam um baixo valor energético (du Toit *et al.*, 2001). O aumento do consumo de frutos e vegetais associa-se à protecção contra diversas doenças, incluindo carcinomas e doenças cardio- e cerebrovasculares (Guthrie & Kurowska, 2001). Esta associação é, com frequência, atribuída aos antioxidantes presentes nestes alimentos, tais como vitaminas C e E, carotenóides, ácidos orgânicos, aminoácidos, ácidos fenólicos e flavonóides, os quais previnem os danos provocados por radicais livres (du Toit *et al.*, 2001).

O marmelo é o fruto de uma árvore decídua da família das Rosáceas, a *Cydonia oblonga* Miller. Apesar deste fruto cru não ser edível, devido à sua dureza, amargura e adstringência, é bastante apreciado pelos Portugueses sob a forma de marmelada.

De acordo com a Legislação Portuguesa (Decreto-Lei n.º 97/84 de 28 de Março), a marmelada é o produto resultante da mistura homogénea e consistente, obtida exclusivamente da cozedura do mesocarpo do marmelo com açúcares. As marmeladas têm deixado de ser confeccionadas de forma artesanal e, quando produzidas industrialmente, apresentam, por vezes, uma fraca qualidade.

Esta dissertação de doutoramento vem na sequência da dissertação de mestrado intitulada "Avaliação da autenticidade de derivados de marmelo através do perfil fenólico" (Silva, 1999a), a qual foi apresentada pela candidata ao grau de Doutor. Até à data de início do trabalho experimental desta dissertação de doutoramento, a investigação efectuada sobre o fruto de *Cydonia oblonga* Miller era reduzida e principalmente direccionada para o estudo da composição das fracções volátil e terpénica não volátil, sendo a primeira a principal responsável pelo seu odor forte e característico (Schreyen *et al.*, 1979; Tsuneya *et al.*, 1980 e 1983; Umano *et al.*, 1986; Ishihara *et al.*, 1983 e 1986; Winterhalter & Schreier, 1988a,b; Winterhalter *et al.*, 1990, 1991a,b e 1999; Guldner & Winterhalter, 1991; Lutz *et al.*, 1991; Lutz & Winterhalter, 1992 e

1993). Alguns investigadores dedicaram-se ainda ao estudo dos polissacarídeos do marmelo, nomeadamente das pectinas do fruto (Forni *et al.*, 1994) e das mucilagens das sementes (Forgács *et al.*, 1998; Vignon & Grey, 1998). Também existiam alguns trabalhos na área dos compostos fenólicos, um deles sobre o isolamento e identificação estrutural de um *O*-$\beta$-glucopiranósido de procianidinas (Porter *et al.*, 1985) e os restantes (resultantes do trabalho experimental da referida dissertação de mestrado) sobre a utilidade da utilização dos perfis fenólicos na avaliação da genuinidade de derivados de marmelo (Andrade *et al.*, 1998 e 1999; Silva, 1999a,b; Silva *et al.*, 2000a,b,c).

Os compostos fenólicos são metabolitos secundários presentes no Reino Vegetal. Pela sua larga e, simultaneamente, diferenciada distribuição por certos grupos botânicos, alguns deles podem ser considerados marcadores químicos da autenticidade dos frutos (Macheix *et al.*, 1990; Spanos & Wrolstad, 1990 e 1992; Spanos *et al.*, 1990; Simón *et al.*, 1992; van Gorsel *et al.*, 1992; Tomás-Lorente *et al.*, 1992; Tomás-Barberán *et al.*, 1993; Oleszek *et al.*, 1994; Vallés *et al.*, 1994; Ooghe *et al.*, 1994a,b; Garcia-Viguera & Bridle, 1995; Ooghe & Detavernier, 1997; Bengoechea *et al.*, 1997; Larrauri *et al.*, 1997; Andrade *et al.*, 1998 e 2000; Silva, 1999a,b; Silva *et al.*, 2000a,b,c).

A autenticidade de um produto pode ser definida como a ausência de adulteração/falsificação, e/ou então como a presença de um conjunto de componentes que lhe são característicos, ou seja, dos seus marcadores químicos. De acordo com Ribéron-Gayon (1968) para que um composto possa ser utilizado como marcador taxonómico deverá obedecer a várias condições:

- não deve pertencer aos constituintes principais universalmente distribuídos;
- não deve ter uma estrutura demasiado complexa, elaborada por um número restrito de espécies;
- deve ser acumulado e, por consequência, intervir de forma limitada no metabolismo;
- deve ser de fácil detecção.

Certos compostos fenólicos reúnem estes requisitos e por isso são utilizados como marcadores de autenticidade.

De facto, é possível associar a certos produtos de origem vegetal um perfil fenólico qualitativo e quantitativo característico que permite garantir a sua autenticidade. Todavia,

convém referir o facto de a produção de compostos fenólicos depender não só da linha biogenética, mas também de vários factores ambientais, tais como a exposição à luz, a temperatura e a humidade (Macheix *et al.*, 1990), o que obviamente deve ser tido em consideração na avaliação de autenticidade por criar dependências geográficas e climáticas.

Além disso, é de considerar a variação existente dentro da própria planta, a qual se pode revelar não só em termos qualitativos (por exemplo, os compostos presentes nas folhas podem ser diferentes daqueles existentes nas raízes, flores ou frutos), mas também em termos quantitativos (apesar de os compostos serem os mesmos, a sua concentração nas diferentes partes da planta pode variar significativamente) (Harborne, 1989). Não havendo, até ao momento, técnica infalível de avaliação da autenticidade, sobretudo nos produtos manufacturados ou industrializados, o recurso a estes compostos constitui uma preciosa ajuda.

Os compostos fenólicos dos frutos têm grande influência nas suas características sensoriais (cor, adstringência e sabor/aroma), as quais podem ser afectadas no decurso dos processos tecnológicos usados na obtenção dos sumos e de outros produtos transformados (Spanos & Wrolstad, 1990 e 1992; Spanos *et al.*, 1990).

A presença de agentes antioxidantes ao nível alimentar é importante, não só para preservação dos alimentos, mas também porque constituem um suplemento de defesa para o organismo (Shi *et al.*, 2001; Valentão, 2002). Entre as diferentes classes de compostos com propriedades antioxidantes, a dos compostos fenólicos representa uma das de maior interesse, dado que, para além de se encontrarem amplamente distribuídos na natureza, apresentam actividade antirradicalar, bem como capacidade para quelatar metais e inibir sistemas geradores de radicais livres (Halliwell *et al.*, 1995; Rice Evans *et al.*, 1995; Shi *et al.*, 2001; Yanishlieva, 2001; Valentão, 2002).

Os ácidos orgânicos são compostos amplamente distribuídos nos frutos (Gomis *et al.*, 1988, Dolenc-Sturm *et al.*, 1999; Shui & Leong, 2002), estando presentes tanto nos frutos crús, como nos seus derivados e muitas vezes aparecem como o resultado de processos fermentativos (Gomis *et al.*, 1988).

Os ácidos orgânicos, para além de serem metabolitos importantes resultantes do metabolismo primário das plantas em geral, desempenham um papel decisivo não só na qualidade dos frutos mas também na dieta humana (Dolenc-Sturm *et al.*, 1999). De facto, a

natureza química, o estado isomérico e a concentração de ácidos orgânicos são factores que influenciam fortemente as propriedades organolépticas dos frutos e dos seus derivados (Lee, 1993). A sua concentração determina o pH e pode afectar o sabor e o aroma dos derivados de frutos e a sua susceptibilidade à degradação microbiana (Gomis *et al.*, 1988). O conteúdo em ácidos orgânicos dos sumos de frutos influencia não só o seu aroma/sabor, mas também a sua estabilidade, o seu valor nutricional e a sua aceitabilidade (Shui & Leong, 2002).

Tem vindo a ser demonstrado que cada fruto tem um perfil em ácidos orgânicos único, o que indica que a análise destes compostos pode ser aplicada na verificação da genuinidade dos derivados de frutos e/ou na determinação dos seus conteúdos em frutos (Coppola & Starr, 1986; Lee, 1993; Câmara *et al.*, 1994; Saavedra *et al.*, 2001; Gokmen *et al.*, 2001; Shui & Leong, 2002). Por exemplo, nos derivados de ananás, a relação ácido cítrico/ácido L-málico constitui um índice de autenticidade, devendo ser próxima de 2 (Câmara *et al.*, 1994). Saavedra *et al.* (2001) demonstraram que a determinação do perfil em ácidos orgânicos permite a avaliação da autenticidade de sumos de laranja. O parâmetro mais adequado para este fim é a relação citrato/isocitrato, que deve ter um valor de 113. A presença de ácido tartárico também parece estar associada à adição de sumo de toranja.

De acordo com Câmara *et al.* (1994), os ácidos orgânicos são dos constituintes dos frutos que apresentam uma menor susceptibilidade de alteração durante o processamento e o armazenamento comparativamente com outros componentes, tais como pigmentos ou compostos responsáveis pelo aroma. Esta relativa estabilidade constitui uma vantagem na utilização do seu perfil como um índice de autenticidade dos derivados de frutos (Evans *et al.*, 1983; Câmara *et al.*, 1994).

A composição em ácidos orgânicos de um fruto pode ser influenciada por diversos factores, tais como a variedade, o grau de maturação, a sua origem geográfica e as condições climáticas nas quais se desenvolve (Gomis *et al.*, 1988; van Gorsel *et al.*, 1992; Blanco *et al.*, 1992; Câmara *et al.*, 1994; Sturm *et al.*, 2003).

O amadurecimento dos frutos envolve uma série de transformações bioquímicas que afectam o conteúdo de diversos compostos que determinam as suas propriedades sensoriais e nutricionais, nomeadamente nos ácidos orgânicos (Blanco *et al.*, 1992). Por exemplo, a concentração de ácidos málico e quínico das maçãs maduras constitui um factor importante na determinação do período óptimo de colheita (Gomis *et al.*, 1988). O decréscimo do teor de ácido

málico, juntamente com a acumulação final de açúcares no final da maturação, parece indicar que este ácido é determinante na gluconeogénese, enquanto que a diminuição do teor de ácido quínico pode ocorrer como consequência da síntese de ácido clorogénico (Blanco *et al.*, 1992).

Os aminoácidos são constituintes fundamentais de toda a matéria viva, estando envolvidos em diversas funções metabólicas, principalmente como "blocos construtores" das proteínas. As suas formas livres participam nas funções celulares, como nutrientes, neurotransmissores e precursores biossintéticos (Chaves das Neves, 1992).

Dentro do grupo das diferentes substâncias que entram na constituição de frutos e vegetais, os aminoácidos têm-se tornado cada vez mais importantes e, por diversas razões, a sua determinação é cada vez mais necessária (Gomis *et al.*, 1990; Fabiani *et al.*, 2002). Isto acontece pois, em primeiro lugar, a concentração de aminoácidos num fruto varia significativamente em função de alterações metabólicas que ocorrem durante o crescimento e a maturação, o que pode ser usado para determinar o melhor período de colheita (Gomis *et al.*, 1990). Em segundo lugar, como os perfis em aminoácidos variam de espécie para espécie e de acordo com a região geográfica de origem, podem ser usados para caracterizar os frutos e os seus derivados (Gomis *et al.*, 1990 e 1992; Belitz & Grosch, 1999). Os aminoácidos podem ainda influenciar a qualidade dos derivados de frutos, uma vez que estes compostos participam em reacções de Maillard e em processos de escurecimento após oxidação enzimática dos polifenóis, factores que determinam a qualidade sensorial de produtos tais como os sumos e as compotas (Gomis *et al.*, 1990).

O perfil em aminoácidos livres é característico de algumas espécies, podendo a sua determinação ser utilizada para averiguar da autenticidade dos derivados de frutos (Ooghe, 1985; Gomis *et al.*, 1990 e 1992; van Gorsel *et al.*, 1992; Robards & Antolovich, 1995; Belitz & Grosch, 1999). Por exemplo, van Gorsel *et al.* (1992) determinaram o perfil em aminoácidos livres de sumos de pêssego, pêra, maçã e uva. O sumo de uva apresenta concentrações em prolina, triptofano e arginina superiores às dos outros sumos, sendo a prolina o aminoácido predominante. O ácido aspártico é predominante nos sumos de pêssego, pêra e maçã estando, no entanto, presente em maiores concentrações no de pêssego. Este sumo apresenta ainda teores em serina maiores do que os restantes. Não foi encontrada tirosina no sumo de maçã, nem metionina no de pêra, o que parece indicar que a presença de tirosina em sumos de maçã ou de metionina

nos de pêra significa uma adulteração por adição de sumo de outros frutos (van Gorsel *et al.*, 1992).

Gomis *et al.* (1992) estudaram o perfil em aminoácidos livres de quatro variedades de maçãs das Astúrias - Collaos, Meana, Picona Rayada e Raxao - ao longo do processo de maturação dos frutos, tendo observado diferenças consideráveis entre os perfis das diferentes variedades. Em três delas (Collaos, Picona Rayada e Raxao), a asparagina foi o aminoácido mais abundante nos frutos colhidos na data óptima de colheita (estimada de acordo com o conteúdo em açúcar, ácidos orgânicos, polifenóis, azoto total e amido de cada variedade). Contudo, na variedade Meana, colhida no mesmo estado de maturação, o aminoácido predominante foi o ácido aspártico. A variedade Raxao apresentou teores em serina inferiores aos encontrados nas outras três variedades. Outras diferenças envolveram aminoácidos presentes em quantidades mais baixas: a variedade Collaos apresentou teores superiores em glutamina, fenilalanina e valina, enquanto que a variedade Picona Rayada apresentou conteúdos mais elevados em alanina. Em termos médios, os aminoácidos mais abundantes nas quatro variedades de maçã, foram a asparagina, os ácidos aspártico e glutâmico e a serina. Estes autores também estudaram a influência do estado de maturação do fruto e verificaram que o teor em asparagina e fenilalanina decresce no final da maturação das quatro variedades. O decréscimo do teor em asparagina pode ser atribuído a uma marcada actividade proteossintética das maçãs durante a maturação, enquanto que a diminuição da concentração em fenilalanina pode dever-se à sua utilização na síntese de antocianinas e de outros compostos fenólicos. A serina foi o único aminoácido livre que sofreu alterações significativas no momento correspondente à data óptima de colheita. Assim, estes autores consideraram que o teor em serina é o único que é útil, como parâmetro complementar, na estimativa do período óptimo de colheita.

O perfil de aminoácidos livres pode ser usado também na autenticação de derivados de citrinos, uma vez que estes frutos são ricos neste grupo de compostos, os quais se apresentam num perfil característico (Robards & Antolovich, 1995). Assim, o conjunto dos oito aminoácidos mais abundantes no sumo de laranja – prolina, ácido aspártico, serina, asparagina, ácido glutâmico, alanina, ácido γ-aminobutírico e arginina – é suficientemente característico para definir este sumo e permitir a detecção de certo tipo de adulterações. Este perfil tem sido usado com êxito na pesquisa de falsificação de sumos de laranja, quer por diluição, quer por adição de sumos de tangerina e de toranja.

Nos vinhos, os aminoácidos livres contribuem para as suas propriedades sensoriais e características finais (Chaves das Neves, 1992). Assim, a composição em aminoácidos é de grande importância na produção de vinho, uma vez que, durante a fermentação, estes compostos constituem uma fonte de azoto para as leveduras (Kosir & Kidric, 2001). Estes compostos são ainda precursores dos álcoois e de outros compostos voláteis. O perfil em aminoácidos livres é influenciado pelo processo de maturação das uvas, pelas condições climáticas, composição dos solos e práticas culturais (Chaves das Neves, 1992).

A presença de D-aminoácidos nos alimentos pode ser devida à isomerização térmica durante o processamento, ou ser resultado de actividade microbiana, sendo particularmente sensível o ácido aspártico, uma vez que é o aminoácido de mais fácil isomerização (Bruckner & Hausch, 1990; Marchelli *et al.*, 1996; Casal, 2004). A sua determinação poderá, então, servir como um indicador de "alimento sobreprocessado", ou ser comprovativo das condições de "boas práticas de fabrico" (Bruckner *et al.*, 1989; Bruckner & Hausch, 1990; Casal, 2004).

De uma forma geral, pode dizer-se que a presença de D-aminoácidos nos alimentos fermentados é natural e superior à existente noutro tipo de alimentos. A D-alanina e os ácidos D-aspártico e D-glutâmico são constituintes normais do peptidoglicano das paredes celulares de microrganismos usados nas fermentações, pelo que não é de estranhar que sejam esses os D-aminoácidos mais frequentemente encontrados nos produtos fermentados (Casal, 2004). O teor e a composição em D-aminoácidos dos alimentos poderão ser úteis para, por exemplo, determinar o processo biotecnológico utilizado na vinificação, bem como a idade de um vinho (Chaves das Neves *et al.*, 1990; Chaves das Neves & Noronha, 1995), o tempo de maturação de um queijo (Marchelli *et al.*, 1997), ou o tipo de culturas de arranque utilizadas no processo fermentativo (Bruckner *et al.*, 1993).

Tendo em consideração o facto do marmelo e do seu principal produto de transformação, a marmelada, serem matrizes pouco estudadas e dada a importância alimentar da marmelada, um produto tradicional Português muito apreciado no nosso País, pareceu-nos ser do maior interesse proceder ao estudo composicional dos mesmos, no sentido de garantir a autenticidade das massas polpadas utilizadas como matéria prima na fabricação e do produto final após manufactura. A escolha recaiu na análise dos compostos fenólicos, como compostos representativos do metabolismo secundário, e dos ácidos orgânicos e dos aminoácidos livres, como compostos

representativos do metabolismo primário das plantas em geral, pois além do seu perfil ser característico de cada espécie, como já foi referido, são compostos amplamente conhecidos quer pelas funções que desempenham na fisiologia e na bioquímica vegetal, quer pela sua acção antioxidante.

## Objectivos

Nesta dissertação de doutoramento pretendeu-se efectuar o estudo do marmelo e da marmelada, no que diz respeito à sua composição em compostos fenólicos, ácidos orgânicos e aminoácidos livres. Para um maior aprofundamento, realizou-se o estudo das três partes individualizadas que constituem o marmelo: a polpa (mesocarpo), a casca (epicarpo) e a semente. Estas duas últimas partes foram individualmente estudadas com o intuito de obter diagramas dos compostos seleccionados, que permitissem através deles detectar fraudes das massas polpadas de marmelo e do produto final da sua transformação industrial. Assim, os principais objectivos foram:

- O aperfeiçoamento do método extractivo de compostos fenólicos em derivados de marmelo utilizado na anterior dissertação de mestrado da autora, através da substituição da resina de Amberlite de XAD-2 por coluna de extracção em fase sólida C18 ISOLUTE (NEC);
- A aplicação da técnica de Ion Trap HPLC-DAD-ESI/MS/MS, ainda pouco praticada no nosso país, à análise do perfil fenólico;
- A validação de metodologias de determinação dos perfis em ácidos orgânicos e aminoácidos livres, por HPLC/UV e GC/FID, respectivamente;
- A aplicação das metodologias validadas às diversas amostras de três anos consecutivos (2000 a 2002): polpas, cascas e sementes de marmelos colhidos em nove zonas geográficas diferentes de Portugal, duas marmeladas caseiras (uma preparada só com polpa de marmelo e outra com marmelos por descascar) e marmeladas de vinte marcas comerciais diferentes;
- O isolamento e identificação estrutural de compostos não identificados nos extractos de polpas e cascas de marmelo, bem como de marmeladas, e a sua posterior quantificação;
- O estudo do efeito do processamento térmico nos diversos constituintes do marmelo;
- A análise estatística multivariada dos resultados obtidos nas amostras de marmelo dos três anos consecutivos de colheita, de forma a avaliar a influência da parte do fruto, da região geográfica de origem e do ano de colheita na sua composição;

- A análise estatística multivariada dos resultados obtidos nas amostras de marmelada, de forma a avaliar o efeito do tipo de processamento (tradicional ou industrial), da marca comercial e do ano de comercialização na sua composição química;
- A avaliação do potencial antirradicalar de extractos metanólicos de polpa, casca e semente de marmelo e de marmelada, utilizando o radical DPPH e respectiva correlação com as fracções fenólica e de ácidos orgânicos, com o objectivo final de aquilatar da importância destes produtos alimentares na saúde, através das suas propriedades antioxidantes.

**PLANO GERAL DA TESE**

## Plano Geral da Tese

A presente dissertação de doutoramento encontra-se estruturada em nove capítulos. A "Parte Experimental" e os "Resultados e Discussão" desta tese, apresentam-se em Inglês, na forma de publicações (Capítulos II a VIII).

No Capítulo I, "Introdução", apresentado em Português, faz-se uma descrição do marmelo e da marmelada, uma abordagem da sua importância económica em Portugal e uma revisão da literatura, no que concerne ao seu valor alimentar/nutricional e aos estudos fitoquímicos previamente publicados.

No Capítulo II, "Perfil em Compostos Fenólicos", são apresentadas três publicações: a primeira é dedicada ao aperfeiçoamento do método extractivo de compostos fenólicos na matriz marmelada; a segunda trata da verificação da aplicabilidade do referido método de extracção a polpas e cascas de marmelo, da sua aplicação a todas as amostras de polpa e casca, colhidas no ano de 2000, e da identificação dos flavonóides característicos da casca recorrendo a uma técnica de Ion Trap HPLC-DAD-ESI/MS/MS; a terceira consiste na definição do perfil fenólico qualitativo de sementes de marmelo, utilizando a mesma técnica.

No Capítulo III, "Isolamento e Identificação Estrutural de Compostos Terpénicos", é apresentado um artigo relativo ao isolamento e à identificação estrutural de compostos terpénicos encontrados pela primeira vez na natureza e que, por isso, foram considerados potenciais marcadores químicos do marmelo e dos seus derivados, bem como à quantificação dos mesmos.

No Capítulo IV, "Perfil em Ácidos Orgânicos", figura uma publicação dedicada à validação de um método analítico de HPLC/UV para a determinação dos ácidos orgânicos presentes em polpas e cascas de marmelo e marmeladas, e a sua aplicação às amostras do ano de 2000.

No Capítulo V, "Perfil em Aminoácidos Livres", são apresentados dois artigos, sendo um deles dedicado ao desenvolvimento e optimização de um método analítico de GC/FID capaz de permitir uma correcta identificação e quantificação dos aminoácidos livres de polpas e cascas de marmelo e de marmeladas; o outro trata da sua aplicação a todas as amostras do ano de 2000. Neste capítulo inclui-se ainda um terceiro artigo, o qual consiste na caracterização da composição fenólica, em ácidos orgânicos e em aminoácidos livres das sementes de marmelo das colheitas efectuadas nos três anos.

No Capítulo VI, "Influência do Processamento Térmico nos Perfis em Compostos Fenólicos, Ácidos Orgânicos e Aminoácidos Livres", é apresentada uma publicação que visa o estudo das alterações que o processamento térmico do marmelo provoca nos diversos compostos estudados.

No Capítulo VII, "Tratamento Estatístico", figuram duas publicações, uma relativa ao tratamento estatístico dos resultados das análises realizadas nas amostras de polpa e casca de marmelo, dos três anos de colheita, e outra relativa à análise estatística dos resultados obtidos nas análises de todas as amostras de marmelada.

No Capítulo VIII, "Actividade Antioxidante", é apresentado um artigo relativo à avaliação do potencial antioxidante de extractos metanólicos de polpa, casca e semente de marmelo e de marmelada caseira, através de um método espectrofotométrico, por monitorização do desaparecimento do radical DPPH.

No Capítulo IX, "Considerações Finais e Conclusões", apresentado em Português, faz-se a sinopse das conclusões mais importantes deste trabalho.

De forma a facilitar a consulta da bibliografia, apresentada de forma dispersa nos diversos artigos, no final desta dissertação de doutoramento é apresentada uma listagem de todas as referências bibliográficas citadas ao longo de todo este texto.

# I. INTRODUÇÃO

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# Introdução

O marmeleiro (*Cydonia oblonga* Miller ou *Cydonia vulgaris* Persoon) é uma planta arbustiva ou subarbórea com ramos penugentos, com folhas ovais e alternas, igualmente penugentas na face inferior (Sampaio, 1947). As grandes flores (Figura 1) brancas ou rosadas aparecem isoladamente sobre curtos pedúnculos e libertam um perfume agradável. Os frutos, marmelos, são pomos amarelos de 10 a 12 cm de diâmetro, muito perfumados, cobertos de penugem (Figura 2). A espécie, originária da região do Cáucaso, difundiu-se progressivamente até à Europa Central e Países Mediterrânicos. Na Antiguidade, o marmeleiro simbolizava a fortuna, a fertilidade e o amor e estava incluído nas plantas medicinais mais estimadas (Volák & Stodola, 1990).

Apesar de perdida a sua antiga reputação, o marmeleiro continua a ser cultivado por toda a Europa, sendo os seus frutos utilizados na preparação de doces, compotas, geleias e xaropes e em fitoterapia (Ivers, 1983; Volák & Stodola, 1990). O marmelo é conhecido como sendo adstringente, emoliente e antidiarreico, propriedades que são atribuídas à presença de taninos e de mucilagens e pectinas (Ivers, 1983; Proença da Cunha *et al.*, 2003).

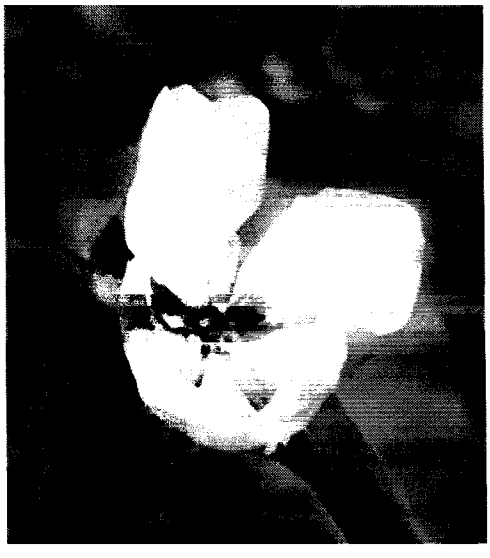

Figura 1 – Flor de marmeleiro.

Figura 2 – Marmelo.

O marmelo maduro tem um forte odor floral; no entanto, o fruto tal qual não é edível devido à sua forte acidez, dureza e adstringência. Em consequência destas características, o marmelo não pode ser consumido sob a forma de sumo, pelo que, em Portugal, é consumido na forma de marmelada, geleia ou cozido em fatias com açúcar.

## 1. Importância Económica do Marmelo e da Marmelada em Portugal

De acordo com as Estatísticas Agro-Industriais de 1999-2001 (INE, 2003a), a actividade da indústria de conservação de frutos e de produtos hortícolas apresentou um crescimento acentuado do valor das vendas no período de 1992 a 2001 (mais 112%), o que equivale a mais de 201 milhões de euros. A classe das indústrias de preparação e conservação de frutos e de produtos hortícolas, na qual está incluída a sub-classe de fabrico de doces de fruta, compotas, geleias e marmeladas, contribuiu com mais de 50% para o valor destas vendas, sendo a actividade mais importante deste grupo (Figura 3).

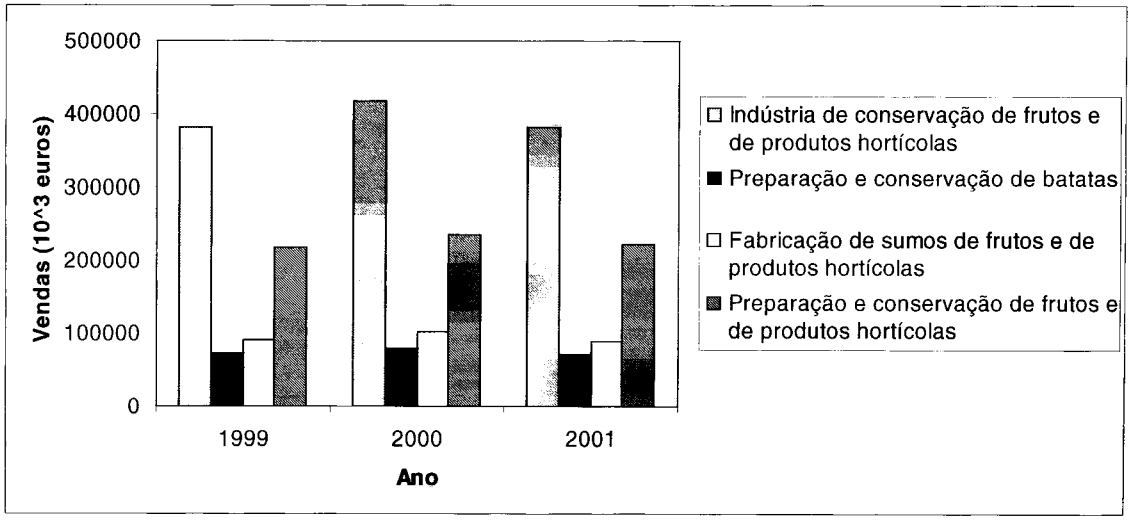


Figura 3 – Valor das vendas da indústria de conservação de frutos e de produtos hortícolas (INE, 2003b).

No período de 1999 a 2001, a marmelada foi o produto mais importante da actividade de fabrico de doces de fruta, compotas, geleias e marmeladas, representando, em 2001, 92% do volume total de produção (Figura 4) e 89% do valor total das vendas (Figura 5).

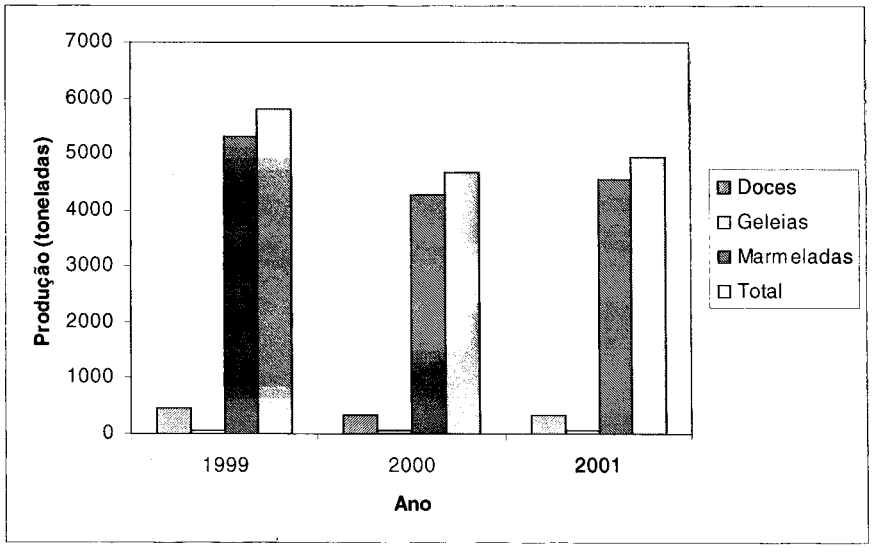


Figura 4 – Fabricação de doces, compotas, geleias e marmeladas – volume de produção vendida (INE, 2003a).

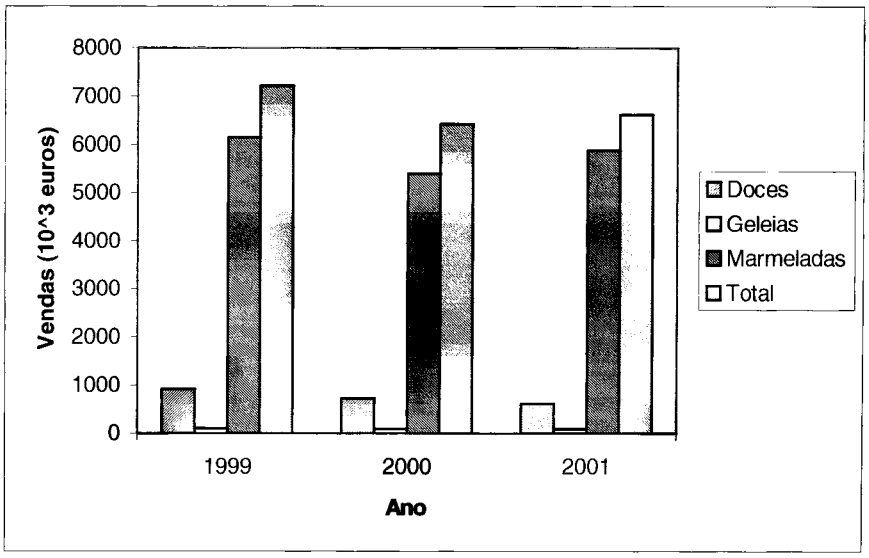


Figura 5 – Fabricação de doces, compotas, geleias e marmeladas – valor da produção vendida (INE, 2003a).

Relativamente à evolução do volume de produção de marmelada no período de 1999 a 2001, verificou-se um decréscimo de 20% no ano de 2000 e uma recuperação de 6% em 2001, ficando, no entanto, abaixo do volume de produção de 1999 (INE, 2003a). Em termos de valor das vendas, houve um decréscimo de 11% no ano de 2000 e um acréscimo de 3% em 2001, evoluindo de forma semelhante ao volume de produção.

A marmelada resulta do processamento térmico da mistura homogénea de polpa de marmelo e açúcar (Gonçalves *et al.*, 1991). Nas marmeladas produzidas industrialmente também são acrescentados aditivos: conservantes (como os ácidos benzóico e sórbico e os respectivos sais), antioxidantes (como o ácido ascórbico), reguladores de acidez (como os ácidos cítrico e tartárico), etc. Tendo em consideração o volume de produção de marmelada, são necessárias grandes quantidades das suas matérias-primas (Figura 6), sobretudo de marmelo e de açúcar, os seus ingredientes maioritários. Por vezes, são utilizadas polpas de marmelo e de outros frutos (Figura 7).

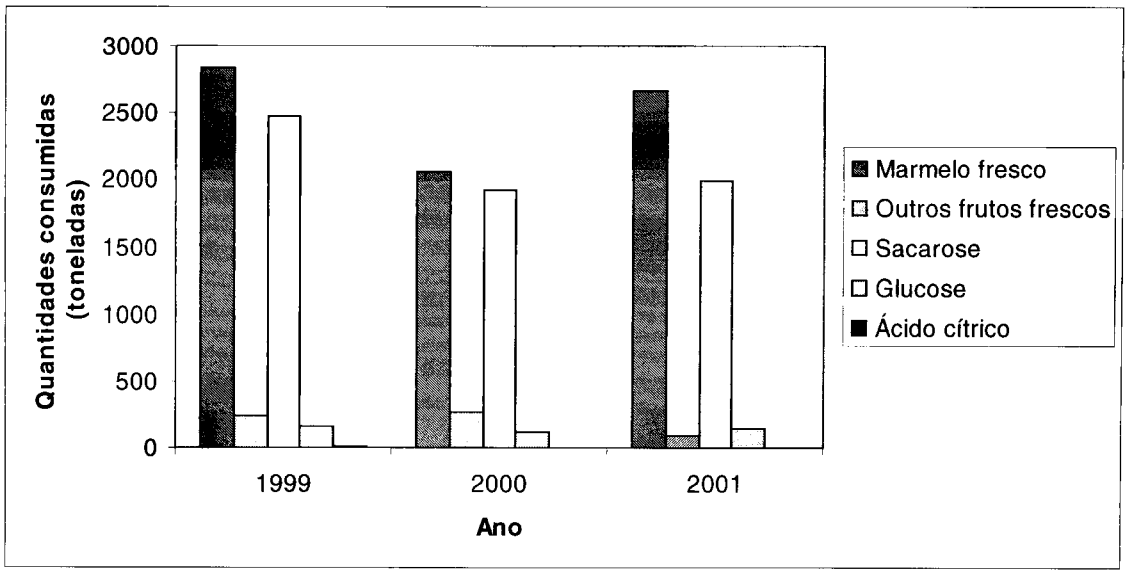


Figura 6 - Fabricação de doces, compotas, geleias e marmeladas - quantidades consumidas das principais matérias-primas (INE, 2003a).

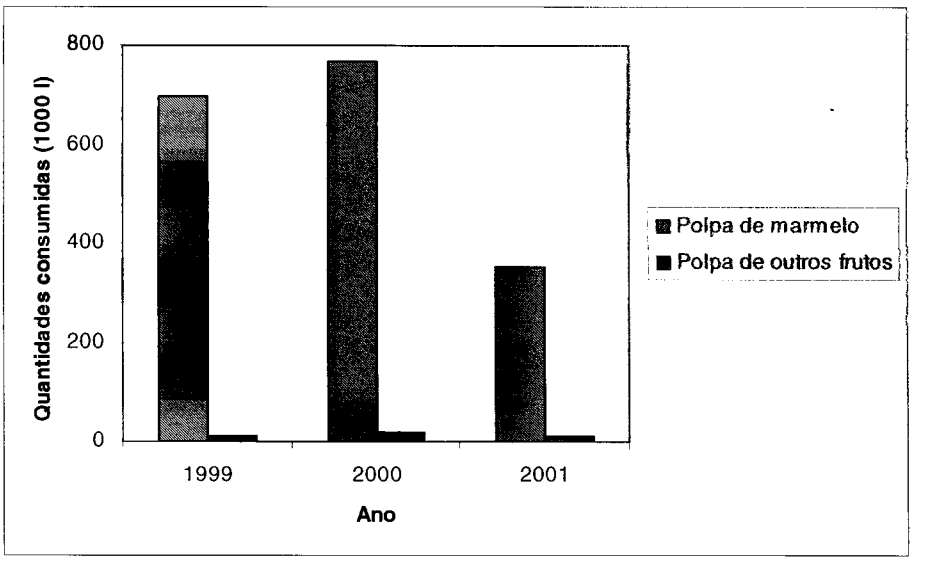


Figura 7 - Fabricação de doces, compotas, geleias e marmeladas - quantidades consumidas de polpas (INE, 2003a).

De acordo com as Estatísticas Agrícolas dos anos de 2000, 2001 e 2002 (INE, 2001, 2002, 2003b), o número de pés de marmeleiro vendidos sofreu um grande aumento no ano de 2001 (Figura 8).

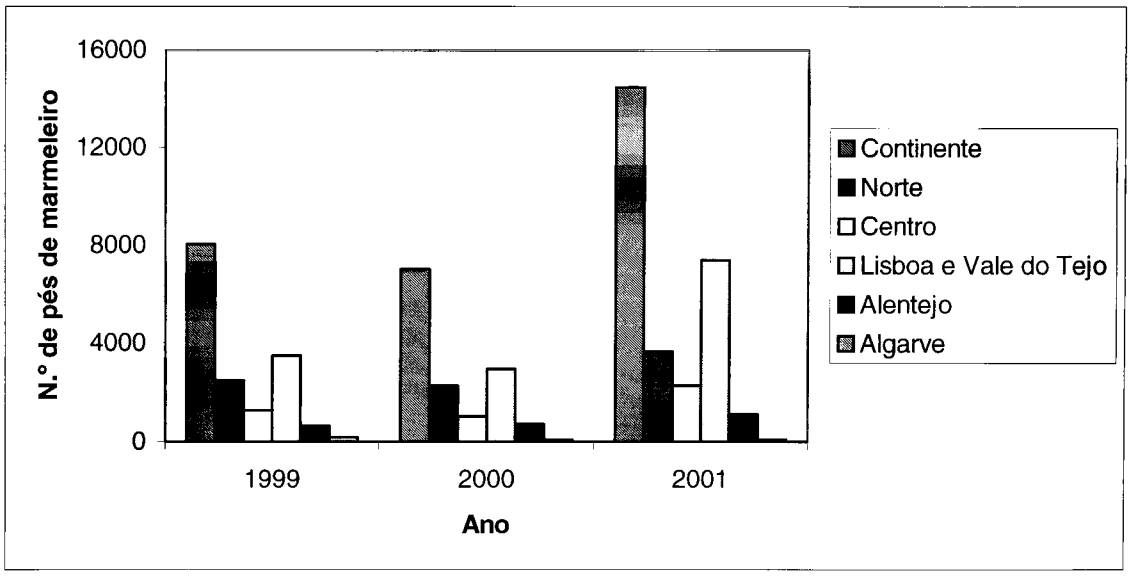


Figura 8 – Número de pés de marmeleiro vendidos pelos viveiristas no Continente (INE, 2001, 2002, 2003b).

Dos dados estatísticos apresentados conclui-se que o marmelo e a marmelada são produtos de elevado consumo em Portugal, uma vez que a marmelada é o produto mais importante da actividade de fabrico de doces de frutos, compotas, geleias e marmeladas e que o marmelo é o seu principal ingrediente.

## 2. Valor Alimentar/Nutricional do Marmelo e da Marmelada

Os frutos frescos são dos alimentos mais atractivos à vista e ao paladar e, tal como os produtos hortícolas, carecem de valor energético importante (25 a 100 kcal / 100 g), praticamente devido a hidratos de carbono, já que são muito pobres em proteínas e gorduras (Gonçalves Ferreira, 1994). Em termos nutricionais, o marmelo é caracterizado por um conteúdo em hidratos de carbono compreendido entre 10 e 15% (Cornatosky, 2000). Na Tabela 1, é apresentada a composição química média, em macro e micronutrientes, da polpa de marmelo.

O marmelo é um fruto com elevado teor em água, de baixo valor calórico (51 a 60 kcal / 100 g), similar ao da maçã (*Malus communis* Lamk) e ao da pêra (*Pirus communis* Lin.), podendo ser utilizado como variante ou alternativa em dietas ou planos alimentares hipocalóricos

ou de conteúdo restrito em hidratos de carbono (em casos de excesso de peso, obesidade, diabetes, ...) (Cornatosky, 2000). O seu conteúdo em proteínas e lípidos é ínfimo.

Tabela 1 – Composição média por 100 g de polpa de marmelo (Cornatosky, 2000).

| Valor calórico e plástico | | | | | |
|---|---|---|---|---|---|
| Água (g) | Hidratos de carbono (g) | Proteínas (g) | Lípidos (g) | Celulose (g) | VCT (kcal) |
| 83-85 | 12-15 | 0,3-0,4 | 0,1 | 1,5-2,2 | 51-60 |
| **Valor mineral (mg)** | | | | | |
| Cálcio | Fósforo | Ferro | Cobre | Sódio | Potássio |
| 6-10 | 17-21 | 0,3-0,6 | 0,13 | 2-9 | 197-203 |
| **Valor vitamínico (mg)** | | | | | |
| Vitamina A | Vitamina $B_1$ | Vitamina $B_2$ | Niacina | Ácido ascórbico | |
| Vestígios | 0,02-0,04 | 0,03 | 0,2-0,7 | 13-15 | |

VCT - valor calórico total.

Em relação aos minerais presentes no marmelo, sobressai o seu considerável conteúdo em potássio (197-203 mg/100g), em valor superior ao da laranja (182 mg/100g), o que lhe confere uma forte acção diurética e estimulante do sistema nervoso (Cornatosky, 2000).

No que diz respeito ao aporte de vitaminas, convém destacar que este fruto é uma boa fonte de ácido ascórbico (vitamina C), tendo, no entanto, valores inferiores aos encontrados nos citrinos.

Para além dos compostos referidos na Tabela 1, a polpa de marmelo contém também, entre outros, aminoácidos livres, óleos essenciais e compostos fenólicos. A presença de taninos confere ao marmelo uma maior acção adstringente (Cornatosky, 2000).

Andrada *et al.* (2003) compararam alguns parâmetros composicionais de marmelos oriundos do Nordeste Argentino (La Rioja, Catamarca e Tucumán) e da região do Cáucaso (Azerbeijão, perto do Mar Cáspio, e Krasnodar, a Norte do Cáucaso) (Tabela 2). De entre os

doze parâmetros estudados, apenas dois, os teores de polifenóis e de ácido ascórbico, evidenciaram diferenças significativas.

Tabela 2 – Parâmetros físico-químicos de marmelos produzidos no Nordeste Argentino e no Cáucaso (Andrada *et al.*, 2003).

| Parâmetros físico-químicos | Região geográfica | |
|---|---|---|
| | Nordeste Argentino | Caúcaso |
| Acidez (% de ácido málico) | 0,76 | 0,77 |
| **pH** | 3,43 | 3,78 |
| °Brix | 13,2 | 15,2 |
| °Brix/acidez | 17,9 | 19,9 |
| Cinzas (%) | 0,45 | 0,43 |
| Pectina (%) | 0,79 | 0,92 |
| Açúcares redutores (%) | 8,31 | 9,27 |
| Açúcares não redutores (%) | 0,69 | 0,85 |
| Açúcares totais (%) | 9,0 | 9,6 |
| Índice de Thiault | 89,3 | 91,7 |
| Polifenóis totais (mg/100g) | 132,2 | 466,3 |
| Ácido ascórbico (mg/100g) | 10,1 | 40,0 |

Segundo a Legislação Alimentar Portuguesa (Decreto-Lei n.º 97/84 de 28 de Março), uma marmelada "normal" deve conter uma quantidade mínima de 400 g de polpa de marmelo por kg de marmelada, subindo este valor para 500 g de polpa por kg de marmelada quando se trata de uma marmelada "extra". Para além disso, o teor em resíduo seco solúvel, determinado por refractometria a 20ºC, deve ser igual ou superior a 60%. Assim sendo, o açúcar é geralmente o principal componente da marmelada, pelo que o consumo deste alimento deve ser moderado. A marmelada é um produto com elevado valor calórico (valor médio aproximado de 272 kcal / 100g), ocupando assim um dos mais altos níveis na escala dos valores energéticos dos alimentos (Vidal, 1938). Este produto integra a lista de alimentos permitidos nas dietas adstringentes, excepto caso hajam restrições calóricas e/ou de hidratos de carbono (Cornatosky, 2000). Na Tabela 3, é apresentada a composição química média de diversas amostras de marmelada provenientes de várias fábricas de Lisboa e de uma amostra do "tipo caseiro" (Vidal, 1938).

Tabela 3 – Composição química média de dezoito amostras de marmelada (Vidal, 1938).

| | Água[a] | Densidade[b] | Extracto seco[a] | Insolúveis[a] | Extracto solúvel[a] | Sacarose[a] |
|---|---|---|---|---|---|---|
| Mínimo | 16,66 | 1,0215 | 61,52 | 2,19 | 59,1 | 19,37 |
| Máximo | 38,48 | 1,0290 | 83,34 | 3,94 | 79,4 | 63,51 |
| Média | 31,09 | 1,0240 | 68,06 | 2,69 | 65,5 | 43,86 |
| SD | 6,002 | 0,0019 | 5,163 | 0,418 | 4,96 | 11,079 |

| | Açúcar invertido[a] | Açúcar total[a] | Azoto[a] | Acidez[c] | Cinzas[a] | Ácido péctico[a] |
|---|---|---|---|---|---|---|
| Mínimo | 4,09 | 56,02 | 0,02 | 4,10 | 0,14 | 0,17 |
| Máximo | 43,97 | 69,51 | 0,05 | 7,80 | 0,49 | 0,83 |
| Média | 18,27 | 62,13 | 0,04 | 5,55 | 0,24 | 0,43 |
| SD | 11,351 | 3,886 | 0,009 | 0,952 | 0,079 | 0,173 |

[a] valores expressos em %; [b] densidade a 20/4 de uma solução aquosa 1:10; [c] valores expressos em $cm^3$ de NaOH N/10 por 100 g.

É também de realçar que na marmelada a concentração de potássio sobe aproximadamente 45%, devido à evaporação da água, com valores a rondar os 290 mg / 100g (Cornatosky, 2000).

## 3. Estudos Fitoquímicos em *Cydonia oblonga* Miller

Até à data de início do trabalho de investigação que aqui se apresenta, poucos estudos fitoquímicos existiam relativamente à espécie *Cydonia oblonga*. Até essa altura, tinham sido estudados os compostos voláteis e terpenos não voláteis do marmelo (Schreyen *et al.*, 1979; Tsuneya *et al.*, 1980 e 1983; Umano *et al.*, 1986; Ishihara *et al.*, 1983 e 1986; Winterhalter & Schreier, 1988a,b; Winterhalter *et al.*, 1990, 1991a,b e 1999; Guldner & Winterhalter, 1991; Lutz *et al.*, 1991; Lutz & Winterhalter, 1992 e 1993; De Tommasi *et al.*, 1996a,b), as pectinas do fruto (Forni *et al.*, 1994), as mucilagens das sementes (Forgács *et al.*, 1998; Vignon & Gey, 1998), alguns compostos fenólicos das folhas de marmeleiro (Polónia, 1957; De Tommasi *et al.*,

1996b) e do marmelo (Porter *et al.*, 1985) e tinha sido estabelecido o perfil fenólico de purés de marmelo, marmeladas e geleias de marmelo (Ferreira *et al.*, 1997; Andrade *et al.*, 1998 e 1999; Silva, 1999a,b; Silva *et al.*, 2000a,b,c).

### *3.1 Compostos voláteis do marmelo*

Os marmelos, quando maduros, têm um sabor/odor muito agradável, forte e duradouro. Foi especialmente na década de 80 que algumas equipas de investigadores se dedicaram ao estudo dos compostos voláteis deste fruto (Schreyen *et al.*, 1979; Tsuneya *et al.*, 1980 e 1983; Ishihara *et al.*, 1983 e 1986; Umano *et al.*, 1986; Winterhalter & Schreier, 1988a,b; Winterhalter *et al.*, 1990; Lutz & Winterhalter, 1992), recorrendo, sobretudo, à GC/MS (Schreyen *et al.*, 1979; Tsuneya *et al.*, 1983; Umano *et al.*, 1986; Winterhalter & Schreier, 1988b; Winterhalter *et al.*, 1990).

Schreyen *et al.* (1979) estudaram o óleo essencial obtido a partir do marmelo (polpa e casca), tendo identificado setenta e nove componentes, em que os ésteres, álcoois, alcanos, cetonas e terpenos foram os compostos maioritários (Tabela 4). O 2-metil-2-butenoato de etilo foi reconhecido por estes autores como sendo determinante para o aroma característico do marmelo.

Mais tarde, como a maior parte do óleo essencial de marmelo é obtido a partir da casca, Umano *et al.* (1986) realizaram o estudo dos compostos voláteis existentes no óleo essencial da casca do marmelo (Tabela 4), utilizando as mesmas metodologias que Schreyen *et al.* (1979), por forma a fazer uma comparação entre a composição química dos dois óleos essenciais. Aqueles autores concluíram que a constituição do óleo essencial da casca é muito diferente da do óleo essencial do fruto completo. Assim, no óleo essencial da casca verificou-se a predominância dos ésteres com pontos de ebulição baixos e que são responsáveis pelo odor floral característico deste fruto. Os ésteres mais abundantes foram o acetato e o propionato de etilo. Os dois principais álcoois presentes na casca foram o etanol e o 2-metilpropanol. Quando comparado com o óleo essencial obtido do fruto inteiro, o da casca apresentou uma proporção consideravelmente menor de álcoois totais/ésteres totais. Foram também encontradas quantidades vestigiais de ácido acético no óleo essencial da casca, o que lhe confere um odor

ligeiramente pungente. Este óleo essencial é ainda diferenciado dos restantes óleos essenciais, pelo seu baixo teor em terpenos, apresentando apenas o *trans*-$\alpha$-farneseno e a $\beta$-ionona.

Um grupo de investigadores japoneses isolou, da fracção volátil do fruto, dois estereoisómeros: as lactonas dos ácidos (2*R*,4*S*)-(+)-2,7-dimetil-4-hidroxi-5(*E*),7-octadienóico e (2*R*,4*R*)-(−)-2,7-dimetil-4-hidroxi-5(*E*),7-octadienóico, considerados por estes autores como os principais responsáveis pelo aroma característico do marmelo (Tsuneya *et al.*, 1980; Ishihara *et al.*, 1983). Como foi a primeira vez que estas lactonas foram isoladas de uma fonte natural, e como se tratava de compostos característicos do óleo essencial de marmelo, foram denominadas de marmelo lactonas ((+)-*trans*- e (−)-*cis*-marmelo lactonas, respectivamente).

Esta mesma equipa, num outro estudo (Tsuneya *et al.*, 1983), identificou sessenta e dois compostos voláteis do óleo essencial do marmelo (dois hidrocarbonetos, treze ésteres, onze álcoois, onze aldeídos, onze cetonas, cinco lactonas e nove outros compostos) (Tabela 4), sendo trinta e cinco deles detectados pela primeira vez. Entre estes destacam-se dois novos óxidos que foram encontrados pela primeira vez na natureza, os estereoisómeros do 3-metil-5-[(*E*)-3'-metil-1',3'-butadien-1'-il]tetrahidrofurano. Por esse motivo, estes óxidos foram designados de marmelo óxidos *cis* e *trans*. Dos sessenta e dois compostos encontrados, o *trans*-$\alpha$-farneseno (31,36%) e as (+)-*trans*- e (−)-*cis*-marmelo lactonas revelaram-se os componentes mais abundantes (10,34 e 13,03%, respectivamente). A presença de *trans*-$\alpha$-farneseno não é de estranhar, uma vez que este é encontrado frequentemente nos óleos essenciais de diversos frutos. A ocorrência dos quatro estereoisómeros da megastigma-4,6,8-trien-3-ona e da teaspirona é pouco comum e a presença de diversos derivados de iononas, com vários tipos de grupos funcionais, parece ser única neste fruto (Tsuneya *et al.*, 1983).

Muitos dos compostos responsáveis pelo forte odor/sabor do marmelo resultam da degradação oxidativa dos carotenóides (Tsuneya *et al.*, 1980 e 1983; Ishihara *et al.*, 1986; Winterhalter & Schreier, 1988a,b; Winterhalter *et al.*, 1990, 1991a,b e 1999; Guldner & Winterhalter, 1991; Lutz & Winterhalter, 1992 e 1993; De Tommasi *et al.*, 1996; Lutz *et al.*, 2002), sendo a maioria pertencente à classe dos norisoprenóides com treze átomos de carbono (Lutz *et al.*, 2002).

Em 1986, Ishihara *et al.* continuaram o estudo dos compostos voláteis do marmelo tendo encontrado o 3,4-didesidro-$\beta$-ionol e quatro novos biciclo[4.3.0]nonanos: o 2,2,6,7-tetrametilbiciclo[4.3.0]nona-4,7,9(1)-trieno, a (+)-2,2,6,7-tetrametilbiciclo[4.3.0]nona-4,9(1)-

dien-8-ona, o (−)-2,2,6,7-tetrametilbiciclo[4.3.0]nona-4,9(1)-dien-8-ol e o (−)-2,2,6,7-tetrametilbiciclo [4.3.0] nona-4,9(1)-dien-7,8-diol (Tabela 4).

De acordo com Winterhalter & Schreier (1988a), os teaspiranos isoméricos detectados por Tsuneya *et al.* (1983) não são compostos voláteis originais do marmelo, mas formam-se sob condições ácidas, ou seja, no pH natural do fruto (pH 3,5-3,8), a partir de um precursor natural sensível a estas condições, o 4-hidroxi-7,8-di-hidro-$\beta$-ionol. Nesse mesmo ano, estes autores também isolaram outros constituintes do sumo de marmelo, através da extracção com uma mistura de pentano/diclorometano (2:1). Nas fracções polares, revelou-se a ocorrência de alguns norisoprenóides com treze átomos de carbono encontrados no marmelo pela primeira vez: 4-hidroxi-$\beta$-ionol, 3-hidroxi-$\beta$-ionol, 4-hidroxi-$\beta$-ionona, 4-oxo-$\beta$-ionol, 3-hidroxi-$\beta$-ionona, 5,6-di-hidroxi-$\beta$-ionona e desidrovomifoliol (Winterhalter & Schreier, 1988b).

Na Tabela 4 encontram-se indicados todos os compostos voláteis até agora identificados no óleo essencial de marmelo.

Tabela 4 – Compostos voláteis encontrados no óleo essencial de marmelo.

| Classe de compostos voláteis | Compostos voláteis |
|---|---|
| Hidrocarbonetos | Benzeno (1, 11) |
| | Tolueno (1, 11) |
| | Trimetilbenzeno (1, 11) |
| | Limoneno# (1, 6, 11) |
| | 3-Careno (1, 11) |
| | 1,2,3-Trimetil-5-(2-propenil)benzeno$ (6, 11) |
| | *trans*-α-Farneseno# (3, 6, 11) |
| | *trans*-β-Farneseno (1, 3, 11) |
| | 2,2,6,7-Tetrametilbiciclo[4.3.0]nona-4,7,9(1)-trieno (5) |
| Ésteres | Acetato de etilo# (1, 6, 11) |
| | Propionato de etilo# (1, 6, 11) |
| | Acetato de propilo (1, 11) |
| | 2-Metilpropionato de etilo (1, 11) |
| | Formiato de 3-metilbutilo (1, 11) |
| | Butirato de etilo# (1, 6, 11) |
| | Acetato de butilo# (1, 6, 11) |
| | 2-Butenoato de etilo (1, 11) |
| | 2-Metilbutirato de etilo# (1, 6, 11) |
| | 3-Metilbutirato de etilo (1, 11) |
| | Acetato de 3-metilbutilo (1, 11) |
| | Hexanoato de metilo (1, 11) |
| | 2-Metil-2-butenoato de etilo (1, 11) |
| | Hexanoato de etilo# (1, 6, 11) |
| | 3-Hexenoato de etilo # (1, 6, 11) |
| | Acetato de (*Z*)-3-hexenilo# (1, 3, 6, 11) |
| | Acetato de (*E*)-2-hexenilo$ (6, 11) |
| | Acetato de hexilo# (1, 6, 11) |
| | 2-Metil-2-butenoato de propilo (1, 11) |
| | 2-Hexenoato de etilo (1, 11) |
| | Monoacetato de hidroquinona (1, 11) |
| | Heptanoato de etilo# (1, 6, 11) |
| | Acetato de benzilo (1, 11) |
| | 3-Metilbutirato de 3-metilbutilo (1, 11) |
| | Acetato de heptilo (1, 11) |

# - compostos que foram identificados tanto no óleo essencial do fruto inteiro como no da casca; $ - compostos que só foram identificados no óleo essencial da casca. (1) Schreyen *et al.*, 1979; (2) Tsuneya *et al.*, 1980; (3) Tsuneya *et al.*, 1983; (4) Ishihara *et al.*, 1983; (5) Ishihara *et al.*, 1986; (6) Umano *et al.*, 1986; (7) Winterhalter & Schreier, 1988a; (8) Winterhalter & Schreier, 1988b; (9) Winterhalter *et al.*, 1990; (10) Lutz & Winterhalter, 1992; (11) Silva, 2000.

Tabela 4 (continuação) – Compostos voláteis encontrados no óleo essencial de marmelo.

| Classe de compostos voláteis | Compostos voláteis |
|---|---|
| Ésteres | 3-Metilbutirato de 2-metilbutilo (1, 11) |
| | Octanoato de metilo (1, 11) |
| | Hexanoato de 2-metilpropilo (1, 11) |
| | Benzoato de etilo (1, 11) |
| | Octanoato de etilo# (1, 6, 11) |
| | Nonanoato de etilo# (1, 6, 11) |
| | Decanoato de etilo# (1, 6, 11) |
| | Dodecanoato de etilo# (1, 6, 11) |
| | Benzoato de 3-metilbutilo (1, 11) |
| | Cinamato de etilo (1, 11) |
| | Caproato de etilo (3) |
| | 3-Hidroxihexanoato de etilo (3) |
| | Caprilato de etilo (3) |
| | Caprato de etilo (3) |
| | Laurato de etilo (3) |
| | *trans*-2-Dodecenoato de etilo (3) |
| | Caproato de *cis*-3-hexenilo (3) |
| | *cis*-4-Decenoato de etilo (3) |
| | *trans*-4-Decenoato de etilo (3) |
| | Caproato de hexilo (3) |
| | Caprilato de hexilo (3) |
| | Ftalato de dibutilo (3) |
| | Diacetilo$ (6, 11) |
| | 2-Metil-2-propenoato de etilo$ (6, 11) |
| | Isobutirato de hexilo$ (6, 11) |
| | Hexanoato de isobutilo$ (6, 11) |
| | Butirato de hexilo$ (6, 11) |
| | 2-Metilbutirato de hexilo$ (6, 11) |
| | Butirato de (*Z*)-3-hexenilo$ (6, 11) |
| | (*E*)-6-Octenoato de etilo$ (6, 11) |
| | (*Z*)-6-Octenoato de etilo$ (6, 11) |
| | 3-Hidroxibutirato de etilo$ (6, 11) |
| | Octanoato de propilo$ (6, 11) |
| | Octanoato de isobutilo$ (6, 11) |
| | (*E*)-6-Nonenoato de etilo$ (6, 11) |
| | (*Z*)-6-Nonenoato de etilo$ (6, 11) |
| | Octanoato de butilo$ (6, 11) |
| | (*E*)-4-Decenoato de etilo$ (6, 11) |
| | (*Z*)-4-Decenoato de etilo$ (6, 11) |
| | (*E*)-7-Dodecenoato de etilo$ (6, 11) |
| | Tetradecanoato de etilo$ (6, 11) |
| | (*E*)-9-Tetradecenoato de etilo$ (6, 11) |
| | (*Z*)-9-Tetradecenoato de etilo$ (6, 11) |
| | Propionato de anisilo$ (6, 11) |
| | Hexadecanoato de etilo$ (6, 11) |

# - compostos que foram identificados tanto no óleo essencial do fruto inteiro como no da casca; $ - compostos que só foram identificados no óleo essencial da casca. (1) Schreyen *et al.*, 1979; (2) Tsuneya *et al.*, 1980; (3) Tsuneya *et al.*, 1983; (4) Ishihara *et al.*, 1983; (5) Ishihara *et al.*, 1986; (6) Umano *et al.*, 1986; (7) Winterhalter & Schreier, 1988a; (8) Winterhalter & Schreier, 1988b; (9) Winterhalter *et al.*, 1990; (10) Lutz & Winterhalter, 1992; (11) Silva, 2000.

Tabela 4 (continuação) – Compostos voláteis encontrados no óleo essencial de marmelo.

| Classe de compostos voláteis | Compostos voláteis |
|---|---|
| Ésteres | Isobutirato de etilo$ (6, 11) |
| | Acetato de isobutilo$ (6, 11) |
| | Propionato de isobutilo$ (6, 11) |
| | Isobutirato de isobutilo$ (6, 11) |
| | 3-Butenoato de etilo$ (6, 11) |
| | Acetato isoamílico$ (6, 11) |
| | Pentanoato de etilo$ (6, 11) |
| | Isobutirato de butilo$ (6, 11) |
| | Butirato de isobutilo$ (6, 11) |
| | 2-Butenoato de etilo$ (6, 11) |
| | Butirato de butilo$ (6, 11) |
| Álcoois | Metanol (1, 11) |
| | Etanol# (1, 6, 11) |
| | 1-Propanol (1, 11) |
| | 2-Metilpropanol# (1, 6, 11) |
| | 1-Butanol# (1, 6, 11) |
| | 3-Metilbutanol (1, 11) |
| | 2-Metilbutanol (1, 11) |
| | 1-Pentanol (1, 11) |
| | 2-Metil-2-buten-1-ol (1, 11) |
| | (*Z*)-3-Hexenol# (1, 3, 6, 11) |
| | (*E*)-2-Hexenol$# (3, 6, 11) |
| | 1-Hexanol# (1, 3, 6, 11) |
| | Fenol (1, 3, 11) |
| | 1-Octanol (1, 11) |
| | 2-Feniletanol (1, 11) |
| | Linalol (1, 3, 11) |
| | Terpinen-4-ol (3) |
| | α-Terpineol (1, 3, 11) |
| | Álcool 2-feniletílico (3) |
| | Di-hidro-β-ionol (3) |
| | Álcool furfurílico (3) |
| | *trans*-Farnesol (3) |
| | Álcool isoamílico$ (6, 11) |
| | Álcool amílico$ (6, 11) |
| | 2-Butanol$ (6, 11) |
| | (−)-2,2,6,7-Tetrametilbiciclo[4.3.0]nona-4,9(1)-dien-8-ol (5) |
| | (−)-2,2,6,7-Tetrametilbiciclo [4.3.0] nona-4,9(1)-dien-7,8-diol (5) |
| | 3,4-Didesidro-*β*-ionol (5) |
| | 4-Hidroxi-7,8-di-hidro-*β*-ionol (7) |
| | 4-Hidroxi-*β*-ionol, (8) |

# - compostos que foram identificados tanto no óleo essencial do fruto inteiro como no da casca; $ - compostos que só foram identificados no óleo essencial da casca. (1) Schreyen *et al.*, 1979; (2) Tsuneya *et al.*, 1980; (3) Tsuneya *et al.*, 1983; (4) Ishihara *et al.*, 1983; (5) Ishihara *et al.*, 1986; (6) Umano *et al.*, 1986; (7) Winterhalter & Schreier, 1988a; (8) Winterhalter & Schreier, 1988b; (9) Winterhalter *et al.*, 1990; (10) Lutz & Winterhalter, 1992; (11) Silva, 2000.

Tabela 4 (continuação) – Compostos voláteis encontrados no óleo essencial de marmelo.

| Classe de compostos voláteis | Compostos voláteis |
|---|---|
| Álcoois | 3-Hidroxi-β-ionol (8) |
| | 4-Oxo-β-ionol (8) |
| | Desidrovomifoliol (8) |
| | Álcool (*S*)-abcísico (10) |
| Aldeídos | Etanal (1, 11) |
| | 3-Metilbutanal (1, 11) |
| | 2-Metilbutanal (1, 11) |
| | 2-Metil-2-butenal (1, 11) |
| | Hexanal# (1, 6, 11) |
| | Furfural# (1, 3, 6, 11) |
| | 5-Metilfurfural (1, 3, 11) |
| | Benzaldeído# (1, 6, 11) |
| | 2,6-Dimetil-2-heptenal (1, 11) |
| | Fenilacetaldeído (3) |
| | *p*-Tolualdeído (3) |
| | Octanal (3) |
| | Nonanal (3) |
| | *trans*-2-*trans*-4-Decadienal (3) |
| | *trans*-2-*cis*-4-Decadienal (3) |
| | Neral (Citral B) (1, 3, 11) |
| | Geranial (Citral A) (1, 3, 11) |
| | *trans*-Farnesal (3) |
| | Heptanal$ (6, 11) |
| | (*E*)-2-Hexenal$ (6, 11) |
| | (*E*)-2-Octenal$ (6, 11) |
| | Acetaldeído$ (6, 11) |
| Cetonas | Acetona# (1, 6, 11) |
| | 2-Butanona (1, 11) |
| | 2-Heptanona (1, 11) |
| | 2-Metil-2-hepten-6-ona (1, 11) |
| | 2-Octanona (1, 11) |
| | β-Ionona# (3, 6, 11) |
| | 5-Nonanona (1, 11) |
| | 2-Nonanona (1, 11) |
| | 2-Decanona (1, 11) |
| | β-Decalona (1, 11) |
| | 2-Pentadecanona (1, 11) |
| | Acetofenona (3) |
| | 6-Metil-5-hepten-2-ona (3) |
| | 3,4-Desidro-β-ionona (3) |

# - compostos que foram identificados tanto no óleo essencial do fruto inteiro como no da casca; $ - compostos que só foram identificados no óleo essencial da casca. (1) Schreyen *et al.*, 1979; (2) Tsuneya *et al.*, 1980; (3) Tsuneya *et al.*, 1983; (4) Ishihara *et al.*, 1983; (5) Ishihara *et al.*, 1986; (6) Umano *et al.*, 1986; (7) Winterhalter & Schreier, 1988a; (8) Winterhalter & Schreier, 1988b; (9) Winterhalter *et al.*, 1990; (10) Lutz & Winterhalter, 1992; (11) Silva, 2000.

Tabela 4 (continuação) – Compostos voláteis encontrados no óleo essencial de marmelo.

| Classe de compostos voláteis | Compostos voláteis |
|---|---|
| Cetonas | Megastigma-4,6,8-trien-3-ona (4 estereoisómeros) (3) |
| | Teaspirona (2 estereoisómeros) (3) |
| | Furilmetilcetona (3) |
| | Metil 5-metilfuril cetona (3) |
| | Acetoína$ (6, 11) |
| | (+)-2,2,6,7-Tetrametilbiciclo[4.3.0]nona-4,9(1)-dien-8-ona (5) |
| | 4-Hidroxi-β-ionona (8) |
| | 3-Hidroxi-β-ionona (8) |
| | 5,6-Di-hidroxi-β-ionona (8) |
| | 4-Hidroxi-7,8-di-hidro-β-ionona (9) |
| | Megastigma-6(*E*),8(*E*)-dien-4-ona (9) |
| Lactonas | *trans*-Marmelo lactona# (2, 3, 4, 6, 11) |
| | *cis*-Marmelo lactona# (2, 3, 4, 6, 11) |
| | γ-Caprolactona (1, 11) |
| | γ-Butirolactona (3) |
| | γ-Valerolactona (3) |
| | γ-Hexalactona (3) |
| | γ-Decalactona$ (6, 11) |
| | γ-Dodecalactona$ (6, 11) |
| Ácidos | Ácido acético$ (6, 11) |
| | Ácido isobutírico$ (6, 11) |
| | Ácido cáprico$ (6, 11) |
| Outros tipos | 2-Acetilfurano (1, 11) |
| | Benzotiazol (1, 11) |
| | *trans*-Marmelo óxido (3) |
| | *cis*-Marmelo óxido (3) |
| | Óxido do *trans*-linalol (3) |
| | Óxido do *cis*-linalol (3) |
| | Vitispirano (3) |
| | (*E*)-Teaspirano# (3, 6, 11) |
| | (*Z*)-Teaspirano# (3, 6, 11) |
| | Eugenol # (3, 6, 11) |
| | Metil eugenol (3) |
| | Éter metílico de eugenilo$ (6, 11) |
| | *p*-Vinilguaiacol (3) |

# - compostos que foram identificados tanto no óleo essencial do fruto inteiro como no da casca; $ - compostos que só foram identificados no óleo essencial da casca. (1) Schreyen *et al.*, 1979; (2) Tsuneya *et al.*, 1980; (3) Tsuneya *et al.*, 1983; (4) Ishihara *et al.*, 1983; (5) Ishihara *et al.*, 1986; (6) Umano *et al.*, 1986; (7) Winterhalter & Schreier, 1988a; (8) Winterhalter & Schreier, 1988b; (9) Winterhalter *et al.*, 1990; (10) Lutz & Winterhalter, 1992; (11) Silva, 2000.

### *3.2 Compostos terpénicos não voláteis do marmelo*

Em 1991, Winterhalter *et al.* e Lutz *et al.* isolaram os precursores glucosídicos das marmelo lactonas e dos marmelo óxidos do sumo de marmelo, os β-D-glucopiranósidos do ácido 2,7-dimetil-8-hidroxi-4(*E*),6(*E*)-octadienóico e do 2,7-dimetil-8-hidroxi-4(*E*),6(*E*)-octadieno, respectivamente. Segundo estes investigadores, as marmelo lactonas e os marmelo óxidos presentes no óleo essencial são compostos resultantes da degradação destes glucósidos por acção de processos tecnológicos, não existindo inicialmente no fruto (Winterhalter *et al.*, 1991a e 1999; Guldner & Winterhalter, 1991; Lutz *et al.*, 1991). Estes autores tiraram estas conclusões uma vez que quando submeteram os extractos glicosídicos de marmelo a temperaturas elevadas, na presença de um pH ácido, natural do fruto, observaram a formação dos referidos compostos (Winterhalter *et al.*, 1991a; Lutz *et al.*, 1991).

No mesmo ano, esta equipa também isolou do marmelo o β-D-genciobiósido [β-D-glucopiranosil(1→6)-β-D-glucopiranósido] do 3-hidroxi-β-ionol (Winterhalter *et al.*, 1991b). De acordo com estes investigadores, este composto é o precursor dos principais norisoprenóides com treze átomos de carbono voláteis do óleo essencial de marmelo (que se formam por acção do calor, a pH ácido natural do fruto), nomeadamente o 2,2,6,7-tetrametilbiciclo[4.3.0]nona-4,7,9(1)-trieno, o 3,4-didesidro-β-ionol e o (−)-2,2,6,7-tetrametilbiciclo[4.3.0]nona-4,9(1)-dien-8-ol, previamente identificados por Ishihara *et al.* (1986).

Na Tabela 5 encontram-se indicados todos os compostos terpénicos não voláteis até agora identificados no marmelo.

Tabela 5 – Compostos terpénicos não voláteis encontrados no marmelo.

| Compostos terpénicos |
|---|
| β-D-glucopiranósido do ácido 2,7-dimetil-8-hidroxi-4(*E*),6(*E*)-octadienóico (1) |
| β-D-glucopiranósido do 2,7-dimetil-8-hidroxi-4(*E*),6(*E*)-octadieno (2) |
| β-D-genciobiósido da (3*R*)-3-hidroxi-β-ionona (3) |
| β-D-glucopiranósido da (3*R*)-3-hidroxi-β-ionona (3) |
| β-D-glucopiranósido da (4*R*,1'*E*,3'*E*)-4-(5'-hidroxi-3'metil-1',3'-pentadienil)-3,5,5-trimetil-2-ciclohexen-1-ona (4) |
| β-D-glucopiranósido do álcool *trans*-abcísico (5) |
| β-D-genciobiósido do 3-hidroxi-β-ionol (6) |

(1) Winterhalter *et al.*, 1991a; (2) Lutz *et al.*, 1991; (3) Guldner & Winterhalter, 1991; (4) Lutz & Winterhalter, 1992; (5) Lutz & Winterhalter, 1993; (6) Winterhalter *et al.*, 1991b.

### *3.3 Compostos terpénicos não voláteis da folha de marmeleiro*

De Tommasi *et al.* (1996a,b) efectuaram estudos nas folhas de marmeleiro, tendo sido isolados e identificados doze glicósidos do $\alpha$- e do $\beta$-ionol (Tabela 6).

Mais tarde, em 2002, Lutz *et al.* isolaram e identificaram mais dois novos compostos das folhas de marmeleiro, os 9-*O*-$\beta$-D-glucopiranósidos do (6*R*)-3-oxo-4-hidroxi-7,8-di-hidro-$\alpha$-ionol e do 3-oxo-5,6-epoxi-$\beta$-ionol (Tabela 6).

Tabela 6 – Compostos terpénicos não voláteis encontrados nas folhas de marmeleiro.

| Compostos terpénicos |
|---|
| 9-*O*-$\beta$-D-apiofuranosil-(1→6)-$\beta$-D-glucopiranósido do 3-oxo-$\alpha$-ionol (1) |
| 9-*O*-$\beta$-D-glucopiranósido do vomifoliol (roseósido) (1) |
| 9-*O*-$\beta$-D-apiofuranosil-(1→6)-$\beta$-D-glucopiranósido do vomifoliol (1) |
| 9-*O*-$\alpha$-L-ramnopiranosil-(1→6)-$\beta$-D-glucopiranósido do blumenol C (2) |
| 9-*O*-$\beta$-D-apiofuranosil-(1→6)-$\beta$-D-glucopiranósido do blumenol C (2) |
| 9-*O*-$\alpha$-L-ramnopiranosil-(1→6)-$\beta$-D-glucopiranósido do blumenol B (2) |
| 9-*O*-$\beta$-D-apiofuranosil-(1→6)-$\beta$-D-glucopiranósido do blumenol B (2) |
| 9-*O*-$\beta$-D-apiofuranosil-(1→6)-$\beta$-D-glucopiranósido do 3$\beta$-hidroxi-$\alpha$-ionol (2) |
| 9-*O*-$\beta$-D-apiofuranosil-(1→6)-$\beta$-D-glucopiranósido do 3$\beta$,6$\alpha$-di-hidroxi-$\alpha$-ionol (2) |
| 9-*O*-$\beta$-D-apiofuranosil-(1→6)-$\beta$-D-glucopiranósido do 7,8-di-hidro-6$\alpha$-hidroxi-$\alpha$-ionol (2) |
| 9-*O*-$\beta$-D-apiofuranosil-(1→6)-$\beta$-D-glucopiranósido do 7,8-di-hidro-3$\beta$,6$\alpha$-di-hidroxi-$\alpha$-ionol (2) |
| 9-*O*-$\beta$-D-glucopiranósido do 7,8-di-hidro-3$\beta$,6$\alpha$-di-hidroxi-$\alpha$-ionol (2) |
| 9-*O*-$\beta$-D-glucopiranósido do (6*R*)-3-oxo-4-hidroxi-7,8-di-hidro-$\alpha$-ionol (3) |
| 9-*O*-$\beta$-D-glucopiranósido do 3-oxo-5,6-epoxi-$\beta$-ionol (3) |

(1) De Tommasi *et al.*, 1996a; (2) De Tommasi *et al.*, 1996b; (3) Lutz *et al.*, 2002.

### *3.4 Polissacarídeos do marmelo*

Como já foi referido anteriormente, o marmelo não é consumido fresco devido à sua acidez e adstringência, sendo usado sobretudo na preparação de marmeladas e de geleias. A caracterização das pectinas extraídas a partir deste fruto permitiu outras utilizações, nomeadamente a de aditivo alimentar.

Forni *et al.* (1994) fizeram a caracterização da pectina solúvel em água ácida quente existente no marmelo inteiro. O rendimento em pectina foi de 0,53% do peso fresco, sendo ligeiramente superior ao da maçã. A pectina do marmelo apresentou um elevado conteúdo em

ácido galacturónico, aproximadamente 78%, um grau de metoxilação de cerca de 59%, correspondendo a uma pectina de grau de metoxilação médio-elevado, e um grau de acetilação de cerca de 6% (características próximas das da pectina da maçã). Assim, este fruto pode ser considerado uma interessante fonte comercial de pectina.

As sementes de marmelo secas contêm até 22% de substâncias mucilaginosas, 15% de óleo, uma pequena quantidade de amigdalósido (bem como da enzima que promove a sua hidrólise, a emulsina) e taninos. Estas sementes são usadas em fitoterapia, em infusão ou em decocção, na proporção de uma a três sementes por chávena de água, contra a tosse, gastrites e diarreias. As sementes esmagadas servem para preparar pensos refrescantes e mucilaginosos, destinados a feridas, inflamações articulares, gretas nos seios ou nas mãos (Volák & Stodola, 1990).

A mucilagem das sementes de marmelo é constituída por uma mistura de celulose e de polissacarídeos solúveis em água (Vignon & Gey, 1998). O principal polissacarídeo hidrossolúvel da mucilagem destas sementes é o 4-*O*-metil-D-glucurono-D-xilano parcialmente *O*-acetilado, com uma proporção de resíduo de ácido glucurónico excepcionalmente elevada. O 4-*O*-metil-D-glucurono-D-xilano isolado da mucilagem de sementes de marmelo foi estudado por Vignon & Gey (1998), recorrendo a $^{13}C$ NMR e $^{1}H$ NMR. Os seus espectros de NMR mostraram que a sua proporção molar de D-xilose e de 4-*O*-metil-D-glucuronato é de 2:1. A mucilagem continha microfibrilas de celulose fortemente ligadas ao glucuronoxilano, possuindo uma proporção de resíduos de ácido glucurónico muito alta. Para além dos resíduos de 4-*O*-metil-α-D-glucopiranosiluronato, a presença de resíduos de α-D-glucopiranosiluronato foi também descrita, na proporção molar de 9:1, respectivamente.

Em 1998, Forgács *et al.* também se dedicaram ao estudo dos polissacarídeos hidrossolúveis destas sementes. Estes autores separaram a mucilagem, extraída com água, em três principais fracções de polissacarídeos: (I) glucano, (II) galactoglucano e manoglucano ou galactomanoglucano (com uma proporção molecular de galactose, manose e glucose de 3:1:9, respectivamente) e (III) arabinoxilano ácido (com uma proporção molar de 1:0,09:0,43:0,14:0,43 de D-xilose, L-arabinose, 4-*O*-metil-D-glucuronato, ácido D-glucurónico e grupos *O*-acetilo, respectivamente).

### *3.5 Compostos fenólicos do marmelo e dos seus derivados*

Os primeiros autores que se dedicaram ao estudo dos compostos fenólicos do marmelo incidiram o seu estudo nos taninos condensados deste fruto. Em 1985, Porter *et al.* isolaram e identificaram um *O*-*β*-D-glucopiranósido de um polímero de procianidinas de marmelos maduros recorrendo a $^{13}C$ NMR. Estes autores verificaram ainda que, no marmelo verde, este polímero não se apresenta glucosilado (Porter *et al.*, 1985).

Mais tarde, Andrade *et al.* (1998) desenvolveram uma metodologia de HPLC/DAD com o objectivo de determinar o perfil fenólico de purés de marmelo, pêra e maçã e verificaram que a referida determinação é útil na avaliação da autenticidade de purés de marmelo e, muito provavelmente, de marmeladas e de outros derivados de marmelo. Foram necessários dois tipos de extracção para a completa definição dos referidos perfis: uma extracção simplificada com metanol e uma extracção com água ácida (pH 2 com HCl), seguida de purificação em coluna de Amberlite de XAD-2. De uma maneira geral, usando este último processo de extracção, a quantidade de cada um dos compostos fenólicos presentes no extracto foi superior. No entanto, a extracção simplificada com metanol foi necessária, uma vez que alguns compostos mais polares, como a arbutina, apresentavam uma taxa de recuperação inferior.

O perfil fenólico do puré de marmelo revelou-se diferente dos de maçã e de pêra, por vezes utilizados para o falsificar, nas épocas em que há escassez de produção de marmelo. Assim, o perfil fenólico do puré de maçã é constituído por (+)-catequina, ácido 5-*O*-cafeoilquínico, (-)-epicatequina, ácido *p*-cumárico, 2'-*O*-xilosilglucosilfloretina, 2'-*O*-glucosilfloretina, rutina, 3-*O*-xilosilquercetina e 3-*O*-ramnosilquercetina (Andrade *et al.*, 1998); enquanto que o puré de pêra contém ácido 3-*O*-cafeoilquínico, (+)-catequina, ácido *p*-hidroxibenzóico, ácido 5-*O*-cafeoilquínico, (-)-epicatequina, 3-*O*-galactosilquercetina, 3-*O*-ramnosilquercetina e arbutina (Andrade *et al.*, 1998). A maçã é caracterizada pela presença de duas di-hidrochalconas, a 2'-*O*-xilosilglucosilfloretina e a 2'-*O*-glucosilfloretina, enquanto que o marcador químico da pêra é a arbutina (Spanos *et al.*, 1990; Spanos & Worlstad, 1990 e 1992; Tomás-Lorente *et al.*, 1992; Tomás-Barberán *et al.*, 1993; Andrade *et al.*, 1998; Silva, 1999a,b; Silva *et al.*, 2000a,b,c). O puré de marmelo foi caracterizado pela presença de ácidos 3-, 4- e 5-*O*-cafeoilquínicos, 3-*O*-galactosilquercetina e rutina (Tabela 7). Em termos quantitativos, no

puré de marmelo o ácido 3-*O*-cafeoilquínico estava presente em quantidade apreciável (cerca de 23%), enquanto que o puré de pêra apenas continha 8% e no de maçã estava ausente. Tal como no puré de pêra, o composto fenólico presente em maior quantidade foi o ácido 5-*O*-cafeoilquínico.

O perfil fenólico dos derivados de fruta, adulterados ou não, tem sido estudado após a utilização de técnicas de separação por HPLC. Como certos compostos fenólicos são característicos de determinados frutos, podem ser usados como marcadores químicos desses frutos (Silva *et al.*, 2000a).

Na sequência do estudo de Andrade *et al.* (1998), surgiu uma tese de mestrado em que a metodologia desenvolvida por estes autores foi aplicada a diversas amostras de marmeladas e geleias de marmelo, caseiras e comerciais, de forma a determinar o seu perfil fenólico e a verificar a sua genuinidade (Silva, 1999a).

Como já foi referido anteriormente, a marmelada é uma compota caseira ou industrial, produzida exclusivamente com polpa de marmelo e açúcar (Decreto-Lei n.º 97/84 de 28 de Março). Em anos de escassa produção de marmelo, a indústria do ramo poderá ter a tentação de usar outros frutos (Ferreira *et al.*, 1997; Andrade *et al.*, 1998). A pêra e a maçã parecem ser as mais utilizadas para o efeito, por serem facilmente acessíveis, de baixo custo e terem uma textura semelhante à do marmelo (Ferreira *et al.*, 1997; Andrade *et al.*, 1998; Silva, 1999a,b; Silva *et al.*, 2000a,b,c). Esta falsificação é facilitada pelo facto do odor forte da marmelada mascarar os odores menos intensos da maçã e da pêra. Assim sendo, a avaliação sensorial falha frequentemente na detecção da referida adulteração.

De acordo com a Legislação Portuguesa (Decreto-Lei n.º 97/84 de 28 de Março e Portaria n.º 497/92 de 17 de Junho), a geleia de marmelo é o produto resultante da mistura de sumo e/ou extracto aquoso de marmelo e de açúcares, em quantidades adequadas, com consistência suficientemente gelificada. Uma geleia de classificação "normal" deve conter uma quantidade mínima de 250 g de marmelo por kg de geleia, subindo este valor para 350 g de fruto por kg de geleia, quando se trata de uma geleia de marmelo do tipo "extra". Para além disso, o teor em resíduo seco solúvel, determinado por refractometria a 20ºC, deve ser igual ou superior a 60%.

Todas as amostras de marmelada apresentaram um perfil fenólico idêntico (procianidina $B_3$, ácidos 3-, 4- e 5-*O*-cafeoilquínicos, 3-*O*-galactosilquercetina e rutina) (Tabela 7), com

predominância de ácido 5-*O*-cafeoilquínico (Silva, 1999a; Silva *et al.*, 2000b). Na maior parte das amostras de marmeladas comerciais encontrou-se arbutina, o que sugere adulteração por adição de pêra. A presença das duas di-hidrochalconas características da maçã não foi detectada. Utilizando esta metodologia foi também possível fazer a determinação simultânea de benzoato de sódio, um conservante bastante utilizado pela indústria alimentar na produção de marmeladas (Andrade *et al.*, 1999; Silva, 1999a; Silva *et al.*, 2000b). A taxa de recuperação deste conservante foi superior nos extractos obtidos através da técnica simplificada com metanol. De uma maneira geral, os teores em benzoato de sódio das marmeladas comerciais foram iguais ou inferiores ao máximo estabelecido na Legislação Portuguesa (Portaria n.º 497/92 de 17 de Junho), ou seja, 1,5 g de ácido benzóico / kg de marmelada (Andrade *et al.*, 1999; Silva, 1999a; Silva *et al.*, 2000b).

Nas amostras de geleia de marmelo o perfil fenólico foi idêntico ao encontrado nas de marmelada (Tabela 7). Neste derivado de marmelo verificou-se a presença de HMF (Silva, 1999a; Silva *et al.*, 2000c), provavelmente, devido ao facto das geleias sofrerem um processamento térmico mais severo, provocando a formação deste composto a partir dos açúcares. Os compostos mais abundantes nas geleias foram o HMF, a procianidina $B_3$ e o ácido 5-*O*-cafeoilquínico. Não foram encontrados os marcadores químicos da maçã nem da pêra, o que indica que não houve adulteração por adição dos referidos frutos (Silva, 1999a; Silva *et al.*, 2000c).

Os resultados destes estudos (Silva, 1999a; Andrade *et al.*, 1999; Silva *et al.*, 2000b,c) indicam que a adulteração de marmeladas tem sido uma prática corrente no nosso País. Como forma de defesa de um produto tradicional Português, seria conveniente que numa Legislação aplicável às marmeladas estivesse incluído um método analítico, designadamente para a verificação do respectivo perfil fenólico, com o objectivo de se poder averiguar da presença ou não de outros frutos, denunciando, assim, eventuais adulterações.

Tabela 7 – Perfil fenólico dos derivados de marmelo
(Andrade *et al.*, 1998; Silva, 1999a,b; Silva *et al.*, 2000a,b,c).

| Classe de compostos fenólicos | Compostos fenólicos |
|---|---|
| Ácidos cinâmicos | ácido 3-*O*-cafeoilquínico |
| | ácido 4-*O*-cafeoilquínico |
| | ácido 5-*O*-cafeoilquínico |
| Flavonóis | 3-*O*-galactosilquercetina |
| | Rutina |
| Procianidinas | Procianidina $B_3$ |

### *3.6 Compostos fenólicos das folhas de marmeleiro*

Nas folhas de marmeleiro foram efectuados estudos por Polónia (1957), tendo sido isolado o metilarbutósido. Posteriormente, De Tomasi *et al.* (1996b) isolaram e identificaram, juntamente com os glicósidos do $\alpha$- e do $\beta$-ionol, quatro glicosil flavonóis: os 7-*O*-$\beta$-D-glucopiranósidos do 7-hidroxi-5'-metoxi-3',4'-metilenedioxiflavonol, do 5',7-di-hidroxi-3',4'-metilenedioxiflavonol e do 4',7-di-hidroxi-3',5'-dimetoxiflavonol e o 7-*O*-$\beta$-D-galactopiranósido do 4',7-di-hidroxi-3',5'-dimetoxiflavonol.

# II. PERFIL EM COMPOSTOS FENÓLICOS

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J. LIQ. CHROM. & REL. TECHNOL., 24(18), 2861–2872 (2001)

# DETERMINATION OF SELECTED PHENOLIC COMPOUNDS IN QUINCE JAMS BY SOLID-PHASE EXTRACTION AND HPLC

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## ABSTRACT

With the purpose of improving an analytical method for the determination of selected phenolic compounds in quince jams, a comparative study of recoveries by two different non-polar sorbents, Isolute C18 non end-capped (NEC) and Isolute C18 end-capped (EC), was performed. Significant differences in the recovery percentages were found. The results suggest that Isolute C18 (NEC) is the most suitable for the recovery of the selected phenolics from quince jams, when 1% of methanol was added to the samples.

The reversed-phase High-Performance Liquid Chromatography/Diode Array Detector (HPLC/DAD) procedure is rapid, sensitive, reproducible, and accurate. The improved sample

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preparation was simple, involving only a C18 Solid-Phase Extraction (SPE) purification step.

The detection limit values for phenolic compounds were between 0.1 and 1.6 μg $mL^{-1}$ and the method was precise. As a general rule, the recovery values were high, except for arbutin.

This technique can also be useful in the evaluation of commercial quince jams genuineness.

## INTRODUCTION

Quince jam is industrially manufactured, or at home, by boiling a mixture of sugar and quince puree (pulp of fruit of *Cydonia oblonga* Miller, var. *maliformis* or *piriformis*) until a convenient texture is obtained (usually to reach 65-72°Brix). However, when quince production is scarce, industry manufacturers are tempted to adulterate quince jam by adding apple (*Malus communis* Lamk) and/or pear (*Pirus communis* Lin.) due to their low cost.

Phenolic compounds are widely distributed in nature and have been successfully used in the determination of the genuineness of some fruit products.(1-7) The main problem in the analysis of phenolics in quince jams is the latter's very high sugar content, which renders the extraction of these metabolites and sample preparation for HPLC analysis difficult. Liquid-liquid partitions produce inconvenient interphases, which do not permit the complete recovery of phenolic compounds. This problem was solved by using the non-ionic polymeric resin Amberlite XAD-2.(8-10)

In previous papers,(8-10) we reported the identification of selected phenolic compounds in quince products (pulps, jams, and jellies) using an Amberlite XAD-2 column chromatography and a simplified extractive technique with methanol, which also allowed the detection of adulterations by the addition of apple and/or pear. However, the extraction *via* Amberlite XAD-2 is a time-consuming technique, unsuitable for routine analysis in quality control determinations.

The aim of the present study was to improve the analytical technique, using C18 SPE columns, to avoid utilising the Amberlite XAD-2 chromatography, in the qualitative and quantitative analysis of selected phenolics in quince jams.

## EXPERIMENTAL

### Quince Jam Samples and Standards

Quince jam samples (A and B) were purchased in the Portuguese market.

The standards were from Sigma (St. Louis, MO, USA) and from Extrasynthése (Genay, France). 3- and 4-*O*-caffeoylquinic acids were not commer-

cially available, so they were prepared by transesterification of 5-*O*-caffeoylquinic acid using tetramethylammonium hydroxide.(11,12) HPLC grade methanol and formic acid were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

### SPE Columns

The Isolute C18 non end-capped (NEC) and Isolute C18 end-capped (EC) SPE columns (50 μm particle size, 60 Å porosity; 10 g sorbent mass/70 mL reservoir volume) were purchased from International Sorbent Technology Ltd (Mid Glamorgan, UK). The chemical structure of the non-polar sorbent Isolute C18 (NEC) consists in C18 silane covalently bonded to the surface of silica. The sorbent Isolute C18 (NEC) is constituted by C18 silane and trimethyl silyl group, covalently bonded to the surface of silica.

### Determination of Sorption Capacity

In order to test the sorption properties of the different sorbents, an aqueous solution of arbutin (0.51 mg $mL^{-1}$), 5-*O*-caffeoylquinic acid (2.5 mg $mL^{-1}$), phloretin 2'-glucoside (0.55 mg $mL^{-1}$), quercetin 3-galactoside (0.30 mg $mL^{-1}$), rutin (0.20 mg $mL^{-1}$), and quercetin 3-rhamnoside (0.065 mg $mL^{-1}$) (pH 2 with HCl)(13) was prepared. Two aliquots (250 mL each) were passed through the different columns. 1% of methanol was added as a wetting agent to a third aliquot (250 mL), which was then applied to a Isolute C18 (NEC) column. Each column was preconditioned with 60 mL of methanol and 140 mL of water (pH 2 with HCl). The phenolic fraction remaining in the column was then eluted with methanol (ca. 50 mL) (until a negative reaction to NaOH 20%). The methanolic extract was evaporated to dryness under reduced pressure (40°C), redissolved in methanol (1 mL), and 20 μL were analysed by HPLC.

### Extraction of Phenolic Compounds from Quince Jams

Each quince jam (ca. 3 g) was thoroughly mixed with water (pH 2 with HCl)(13) until complete extraction of the phenolic compounds (negative reaction to NaOH 20%), and filtered through cotton wool. 1% methanol was added to the filtrate, which was then passed through an Isolute C18 (NEC) column, preconditioned as described previously. Sugars and other polar compounds were eluted with the aqueous solvent. The retained phenolic fraction was then eluted with methanol (ca. 50 mL) (until negative reaction to NaOH 20%). The extract was

concentrated to dryness under reduced pressure (40°C), redissolved in methanol (1 mL). and 20 µL were analysed by HPLC.

### HPLC Analysis of Phenolic Compounds

Separation of the selected phenolics was achieved as reported previously,(8-10) with an analytical HPLC unit (Gilson), using a Spherisorb ODS2 (25.0 x 0.46 cm; 5 µm, particle size) column. The solvent system used was a gradient of water-formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15%B at 3 min, 25%B at 13 min, 30%B at 25 min, 35%B at 35 min, 45%B at 39 min, 45%B at 42 min, 50%B at 44 min, 55%B at 47 min, 70%B at 50 min, 75%B at 56 min, and 80%B at 60 min, at a solvent flow rate of 0.9 mL/min. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range 200-400 nm, and chromatograms were recorded at 280 and 350 nm. The data were processed on an Unipoint® system software (Gilson Medical Electronics, Villiers le Bel, France).

The compounds in each sample were identified by comparing their retention times and UV-Vis spectra in the 200-400 nm range with the library of spectra previously compiled by the authors. Peak purity was checked by means of the Gilson 160 SpectraViewer Software Contrast Facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3- and 4-*O*-caffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid, phloretin 2'-xylosylglucoside as phloretin 2'-glucoside and quercetin 3-xyloside as quercetin 3-rhamnoside. The other compounds were quantified as themselves.

## RESULTS AND DISCUSSION

### Sorption Capacity Study

The results of sorption capacity for the selected phenolic compounds are summarised in Table 1.

### Analytical Curves and Detection Limits

Under the assay conditions described, a linear relationship (Table 2) between the concentration of arbutin and phloretin 2'-glucoside and the UV absorbance at 280 nm was obtained, as it happened with 5-*O*-caffeoylquinic acid,

***Table 1.*** Phenolic Compounds Recoveries (%) by ISOLUTE C18 Columns

| | Phenolic Compounds | | | | | |
|---|---|---|---|---|---|---|
| SPE Column | Arbutin | 5-O-Cafeoylquinic Acid | Phloretin 2'-Glucoside | Quercetin 3-Galactoside | Rutin | Quercetin 3-Rhamnoside |
| EC | 14.3 | 63.2 | 85.2 | 53.5 | 62.5 | 46.5 |
| NEC | 22.4 | 77.0 | 86.8 | 58.7 | 71.5 | 51.0 |
| NEC* | 18.5 | 95.3 | 105.2 | 100.4 | 100.1 | 88.5 |

EC– end-capped; NEC– non end-capped; *addition of 1% methanol.

quercetin 3-galactoside, rutin, and quercetin 3-*r*hamnoside and the UV absorbance at 350 nm. The correlation coefficient for the standard curves invariably exceeded 0.99. The calibration curves (Table 2) were obtained by triplicate determinations of each of the calibration standards, the peak area values (arbitrary units) were plotted as average values. The relative percent average deviations of triplicates were less than 2% in all cases. The detection limit values were calculated as the concentration corresponding to three times the standard deviation of the background noise.

## Validation of the Method

Results from quantification of the selected phenolic compounds applied to quince jam samples are shown in Table 3. Figure 1 shows the HPLC profile of a quince jam sample, obtained by C18 SPE extraction. The retention times (RT) obtained for phenolic compounds are presented in Table 4.

***Table 2.*** Equations for Regression Lines and Correlation Coefficients, Concentration Range of Linearity and Detection Limits for Phenolic Compounds

| Phenolic Compounds | Equation | Linearity ($\mu g\ mL^{-1}$) | Detection Limit ($\mu g\ mL^{-1}$) |
|---|---|---|---|
| Arbutin | y* = 4,83×106x r = 0.99861 | 134–1070 | 1.6 |
| 5-*O*-Caffeoylquinic acid | y = 2,59×107x r = 0.99735 | 50–5000 | 0.3 |
| Phloretin 2'-glucoside | y* = 5,91×107x r = 0.99997 | 4–500 | 0.1 |
| Quercetin 3-galactoside | y = 4,62×107x r = 0.99823 | 5–600 | 0.2 |
| Rutin | y = 3,06×107x r = 0.99763 | 3–400 | 0.3 |
| Quercetin 3-*r*hamnoside | y = 3,03×107x r = 0.99992 | 1–130 | 0.3 |

y – peak area at 350 nm; y* - peak area at 280 nm; x - μg of phenolic compound; r – correlation coefficient.

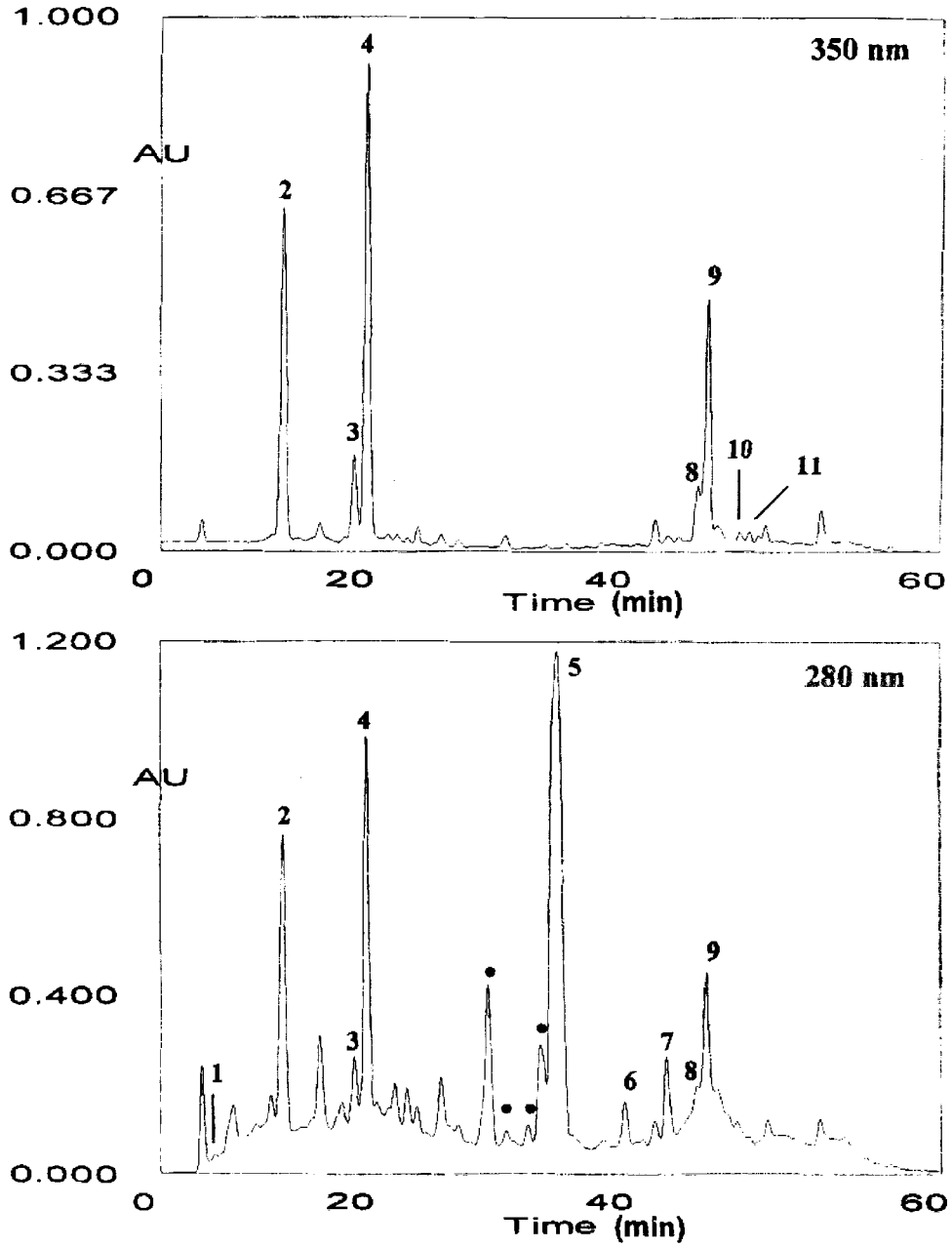


***Figure 1.*** HPLC phenolic profile of a quince jam sample, obtained by C18 SPE extraction. (1) arbutin; (2) 3-*O*-caffeoylquinic acid; (3) 4-*O*-caffeoylquinic acid; (4) 5-*O*-caffeoylquinic acid; • unidentified phenolic compounds (under study); (5) sodium benzoate; (6) phloretin 2'-xylosylglucoside; (7) phloretin 2'-glucoside; (8) quercetin 3-galactoside; (9) rutin; (10) quercetin 3-xyloside; (11) quercetin 3-rhamnoside.

***Table 3.*** Phenolic Composition of Quince Jam Samples (mg $Kg^{-1}$)[a] (Quantification by External Standard Techniques)

| | Samples | |
|---|---|---|
| Phenolic Compounds | A | B |
| 3-*O*-Caffeoylquinic acid | 44.8 (2.49) | 48.7 (0.56) |
| 4-*O*-Caffeoylquinic acid | 13.3 (0.12) | 15.8 (0.40) |
| 5-*O*-Caffeoylquinic acid | 113.4 (4.61) | 102.5 (1.86) |
| Phloretin 2'-xylosylglucoside | 9.3 (0.23) | — |
| Phloretin 2'-glucoside | 20.2 (0.38) | — |
| Quercetin 3-galactoside | 5.1 (0.02) | 12.4 (0.16) |
| Rutin | 39.1 (0.57) | 47.5 (1.17) |
| Quercetin 3-xyloside | 1.1 (0.10) | 0.5 (0.01) |
| Quercetin 3-rhamnoside | 2.9 (0.03) | 0.9 (0.04) |

[a]Values are expressed as mean (standard deviation) of three determinations; A and B – commercial quince jams from different origins.

The precision of the analytical method was evaluated by measuring the peak chromatographic area of phenolic compounds six times on the same sample. The standard deviations and the coefficients of variation (%) of these compounds are presented in Table 5.

In order to study the recovery of the procedure, one quince jam sample was added to known quantities of arbutin, 5-*O*-caffeoylquinic acid, phloretin 2'-glucoside, and rutin (Table 6). The sample was analysed in triplicate before and after the additions. Recovery values were between 2.9 and 12.6 % for arbutin, 82.2

***Table 4.*** Retention Times of the Phenolic Compounds (n = 3)

| Phenolic Compounds | Retention Time[a] (min) | SD (min) |
|---|---|---|
| Arbutin | 4.22 | 0.03 |
| 3-*O*-Caffeoylquinic acid | 9.55 | 0.07 |
| 4-*O*-Caffeoylquinic acid | 15.10 | 0.05 |
| 5-*O*-Caffeoylquinic acid | 16.10 | 0.10 |
| Phloretin 2'-xylosylglucoside | 36.38 | 0.04 |
| Phloretin 2'-glucoside | 39.70 | 0.09 |
| Quercetin 3-galactoside | 42.07 | 0.11 |
| Rutin | 42.83 | 0.08 |
| Quercetin 3-xyloside | 45.43 | 0.06 |
| Quercetin 3-rhamnoside | 46.13 | 0.13 |

[a]Mean of the retention times; SD – standard deviation.

***Table 5.*** Evaluation of the Analytical Method Precision (n = 6) (Quantification by External Standard Techniques)

| Phenolic Compounds | SD (mg $Kg^{-1}$) | CV (%) |
|---|---|---|
| 3-*O*-Caffeoylquinic acid | 0.56 | 1.15 |
| 4-*O*-Caffeoylquinic acid | 0.40 | 2.55 |
| 5-*O*-Caffeoylquinic acid | 1.86 | 1.81 |
| Phloretin 2'-xylosylglucoside | 0.23 | 2.49 |
| Phloretin 2'-glucoside | 0.38 | 1.89 |
| Quercetin 3-galactoside | 0.16 | 1.30 |
| Rutin | 1.17 | 2.45 |
| Quercetin 3-xyloside | 0.01 | 2.72 |
| Quercetin 3-rhamnoside | 0.04 | 3.76 |

SD – standard deviation; CV – coefficient of variation.

***Table 6.*** Recoveries of Arbutin, 5-*O*-Caffeoylquinic Acid, Phloretin 2'-Glucoside and Rutin from a Spiked Quince Jam Sample (Quantification by External Standard Techniques)

| Phenolic Compounds | Present (mg $Kg^{-1}$) | Added (mg $Kg^{-1}$) | Found* (mg$Kg^{-1}$) | SD (mg $Kg^{-1}$) | CV (%) | Recovery (%) |
|---|---|---|---|---|---|---|
| | | 62.5 | 8.8 | 0.59 | 6.66 | 12.6 |
| Arbutin | 7.4 | 165.0 | 8.9 | 0.90 | 10.17 | 5.2 |
| | | 331.1 | 9.9 | 0.48 | 4.84 | 2.9 |
| | | 199.3 | 262.2 | 16.25 | 6.20 | 83.9 |
| 5-*O*-Caffeoylquinic | 113.4 | 436.2 | 459.8 | 25.22 | 5.48 | 83.7 |
| acid | | 866.7 | 805.9 | 24.72 | 3.07 | 82.2 |
| | | 165.6 | 179.9 | 8.89 | 4.94 | 96.8 |
| Phloretin | 20.2 | 250.0 | 240.3 | 0.73 | 0.30 | 88.9 |
| 2'-glucoside | | 312.5 | 298.8 | 2.22 | 0.74 | 89.8 |
| | | 66.2 | 100.2 | 0.08 | 0.08 | 95.2 |
| Rutin | 39.1 | 132.9 | 138.3 | 3.28 | 2.37 | 80.4 |
| | | 234.9 | 212.2 | 3.66 | 1.72 | 77.4 |

*Mean value found for three assays for each studied concentration; SD – standard deviation; CV – coefficient of variation.

and 83.9 % for 5-*O*-caffeoylquinic acid, 88.9 and 96.8 % for phloretin 2'-glucoside, and between 77.4 and 95.2 % for rutin.

## Sorption Capacity Study

SPE is a very simple technique, employing inexpensive, disposable extraction columns that are available in a multitude of column sizes and sorbents. Due to the fact that phenolics are acidic compounds, our first choice was a C18 SPE column, once these sorbents provide enhanced retention of this kind of compound. As can be seen in Table 1, the recovery of the selected phenolic compounds from the sorbent is higher using the Isolute C18 (NEC) column. This can be justified by the fact that this sorbent has secondary silanol interactions associated with surface silanol groups (non-polar, polar and cation exchange) compared to Isolute C18 (EC). As a general rule, adding 1% methanol to the sample, as A wetting agent, we could increase the percentage of recovery of the selected phenolics. This addition was required in order to maintain an active sorbent surface, once we were dealing with large volume samples (ca. 750 mL). This last procedure was applied to quince jam samples.

The flow rate was controlled and maintained in all determinations, to ensure reproducibility.

## Analytical Curves and Detection Limits

In previous papers(8-10) we reported the presence of 3-, 4- and 5-*O*-caffeoylquinic acids, unidentified phenolic compounds (under study), quercetin 3-galactoside, rutin, quercetin 3-xyloside, and quercetin 3-rhamnoside in quince jams. When these products were adulterated by addition of pear and/or apple, they also contained arbutin (characteristic compound of pear), phloretin 2'-xylosylglucoside, and phloretin 2'-glucoside (chemical markers of apple). For these reasons, calibration curves, concentration ranges of linearity, and detection limits of all of these phenolic compounds were now determined (Table 2).

In order to minimise the quantification errors, quercetin-3-galactoside and rutin standards were always injected simultaneously.

## Validation of the Method

The selected phenolics from two quince jam samples were analysed by the proposed technique (Table 3), in order to validate this procedure and assess its application to the routine phenolic analysis of quince jams.

In previously published material(8-10) we have identified two quercetin glycosides as rutin and quercetin 3-galactoside, rutin having a lower RT than quercetin 3-galactoside, which agrees with several authors;(1,7) but now we have deduced that this correlation is not correct, and quercetin 3-galactoside, in reality, is the compound with lower RT. We have come to this conclusion by comparing the RT of these compounds with different blends of standards of rutin and quercetin 3-galactoside from Extrasynthese. We have tested two columns, Spherisorb ODS2 (25.0 x 0.46 cm; 5 μm, particle size) and Hypersil ODS (20.0 x 0.40 cm; 5 μm, particle size) for the separation of these standards. We have tried several gradients and flows. In all cases, quercetin 3-galactoside was the compound with lower RT. One would expect quercetin 3-rhamnosylglucoside (rutin) to have a smaller RT than the quercetin 3-galactoside. However, according to Casteele et al. (1982), the glycosylation of an OH group may (due to hydrogen linkages or steric hindrance) hinder some of the hydrophilic moieties of the molecule.(14)

The analytical method is precise, once the coefficients of variation of phenolics were between 1.15 and 3.76 % (n = 6) (Table 5).

Given the similarity of chemical structures between the several caffeoylquinic acids, phloretin, and quercetin glycosides and, therefore, their UV spectra and absortivity, the recoveries of the extractive method were only determined for arbutin, 5-*O*-caffeoylquinic acid, phloretin 2'-glucoside, and rutin (Table 6). This procedure demonstrated the effectiveness of the extraction and the accuracy of the proposed method, except for arbutin.

The phenolic profile obtained with the developed SPE procedure (Figure 1) is similar to that obtained in previous works with Amberlite XAD-2 and methanolic extractions.(8-10) In those studies, arbutin was only detected in the extracts obtained with a simplified technique using methanol as extractive solvent. The presence of arbutin in the SPE extracts can be explained by the combination of both strong primary (non-polar) and secondary (silanol) interactions, which allows some retention of polar compounds. However, when this extractive procedure was used, arbutin presented low recover rates, which could be due to the polarity of this compound, allowing its elution with sugars and other polar compounds. So, the simplified technique is also needed for the quantification of arbutin, in adulterated quince jams. This method is suitable for detection but not for quantification of arbutin.

One of the analysed samples presented, the dihydrochalcones phloretin 2'-xylosylglucoside and phloretin 2'-glucoside, considered the chemical markers of apple, suggests a problem with this fruit.

The extraction with Amberlite XAD-2 presents some disadvantages. It is a time-consuming technique, unsuitable for routine analysis in quality control determinations. Additionally, the chromatograms obtained were not so clean as those from SPE extraction, which shows that this new extraction technique is

more suitable for phenolics analysis. Finally, Amberlite XAD-2 is rather expensive, although it can be reutilized for a large number of assays.

The main advantages of the SPE method herein developed, are that it presents highly purified extracts, good recoveries of analytes, high reproducibility, and requires small amounts of sample. The analytical procedure is simple and the costs of experiments low.

In conclusion, the proposed reversed-phase HPLC procedure for phenolic profile determination is simple, rapid, sensitive, reproducible, and accurate, and is suitable for routine analysis of phenolics in quince jams. The utilisation of C18 (NEC) SPE columns avoids the use of Amberlite XAD-2 resin, which is a rather time consuming technique. This procedure also allows the detection of adulterations by addition of apple and/or pear; however, it can't be used in the quantification of arbutin (marker of pear).

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# Phenolic Profile of Quince Fruit (*Cydonia oblonga* Miller) (Pulp and Peel)

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Qualitative and quantitative analyses of phenolic compounds were carried out on quince fruit samples from seven different geographical origins in Portugal. For each origin, both pulp and peel were analyzed by reversed-phase HPLC-DAD and HPLC-DAD/MS.The results revealed differences between the phenolic profiles of pulps and peels in all studied cases. The pulps contained mainly caffeoylquinic acids (3-, 4-, and 5-*O*-caffeoylquinic acids and 3,5-dicaffeoylquinic acid) and one quercetin glycoside, rutin (in low amount). The peels presented the same caffeoylquinic acids and several flavonol glycosides: quercetin 3-galactoside, kaempferol 3-glucoside, kaempferol 3-rutinoside, and several unidentified compounds (probably kaempferol glycoside and quercetin and kaempferol glycosides acylated with *p*-coumaric acid). The highest content of phenolics was found in peels.

KEYWORDS: ***Cydonia oblonga* Miller; quince fruit; HPLC-DAD; HPLC-DAD/MS; phenolics**

## INTRODUCTION

Quince fruit (*Cydonia oblonga* Miller) is too acid, astringent, and tough to be consumed fresh. However, it can be consumed when cooked or processed as jam or jelly, two food products with great tradition in Portugal. Quince is a seasonal fruit and its homemade jam and jelly are prepared during September and October. According to the Portuguese Legislation, quince jam is the food product of the homogeneous and consistent mixture, obtained exclusively by boiling quince mesocarp with sugars, and quince jelly is the product obtained by cooking a mixture of juice and/or aqueous extract of quince fruit and sugars, in appropriate amounts, with sufficient jellied consistency (*1*).

Phenolic compounds constitute a large and heterogeneous class of compounds with a very wide distribution in taxa of higher plants. Despite this almost ubiquity, experimental evidence has demonstrated that each plant species is characterized by the presence of a limited number of compounds. Within each species, the nature of these compounds can vary from organ to organ but is constant enough toward several other factors. These facts have been used, in recent years, in the characterization of several food products of plant origin by their phenolic profile. Factors contributing to the variability in phenolic distribution include cultivar and genetics, geographical origin, maturity, climate, position on tree, and agricultural practices (*2*).

For quince fruit and its derivatives, few studies have been developed. The usefulness of phenolic compounds in the determination of genuineness of quince puree (*3*), jam (*4*, *5*), and jelly (*6*) has been reported. Recently, an HPLC-UV method was developed for the determination of organic acids in quince fruit and its jam (*7*). As far as we know, there are few studies about phenolic composition of quince fruit. Glucosides of procyanidin polymers have been previously identified in this fruit (*8*, *9*). The work herein represents a contribution for the chemical characterization of pulp and peel from this fruit. With this purpose, samples of seven different geographical origins from Portugal were analyzed.

## MATERIALS AND METHODS

**Samples.** Healthy quince fruit samples were collected in different places in Northern (Amarante, Baião, Vila Real, and Bragança) and Central Portugal (Viseu, Pinhel and Covilhã), in the year 2000. All fruits were separated into pulp and peel. Each part of the fruit was cut in thin slices and freeze-dried. Lyophilizations were carried out using a Labconco 4.5 apparatus (Kansas City, MO).

**Standards.** The standards were from Sigma (St. Louis, MO) and Extrasynthèse (Genay, France). 3- and 4-*O*-Caffeoylquinic acids were not commercially available, so they were prepared by transesterification of 5-*O*-caffeoylquinic acid (chlorogenic acid) using tetramethylammonium hydroxide (*10*, *11*). Methanol, and hydrochloric and formic acids were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

**Solid-Phase Extraction (SPE) Columns.** The ISOLUTE C18 non end-capped (NEC) SPE columns (50-$\mu$m particle size, 60 Å porosity; 10 g sorbent mass/70 mL reservoir volume) were purchased from International Sorbent Technology Ltd. (Mid Glamorgan, UK).

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**Extraction of Phenolic Compounds.** The extraction of phenolics was achieved as previously reported (*5*): each sample (ca. 1 g) was thoroughly mixed with water (pH 2 with HCl) until complete extraction of the phenolic compounds (negative reaction to NaOH 20%) and filtered. 1% Methanol was added to the filtrate, which was then passed through an ISOLUTE C18 (NEC) column which had been preconditioned with 60 mL of methanol and 140 mL of water (pH 2 with HCl). Sugars and other polar compounds were eluted with the aqueous solvent. The retained phenolic fraction was then eluted with methanol (ca. 50 mL). The extract was concentrated to dryness under reduced pressure (40 °C) and redissolved in methanol (1 mL).

**HPLC-DAD/MS System for Qualitative Analysis.** Chromatographic separation was carried out on a LiChroCART column (250 × 4 mm, RP-18, 5-$\mu$m particle size, Merck, Darmstadt, Germany) using two solvents: water/formic acid (19:1) (A) and methanol (B); starting with 5% methanol and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% B at 42 min, 50% B at 44 min, 55% B at 47 min, 70% B at 50 min, 75% B at 56 min, and 80% B at 60 min. The flow rate was 0.9 mL/min, and the injection volume was 80 $\mu$L. Detection was carried out at 270, 320, and 350 nm.

The HPLC system was equipped with a DAD and mass detector in series (Agilent 1100 Series LC/MSD Trap). It consisted of an Agilent G1312A HPLC binary pump, an Agilent G1313 A autosampler, an Agilent G1322A degasser, and an Agilent G1315B photodiode array detector controlled by Agilent software v. A.08.03 (Agilent Technologies, Waldbronn, Germany). The mass detector was an Agilent G2445A ion-trap mass spectrometer equipped with an electrospray ionization (ESI) system and controlled by Agilent Software v. 4.0.25. Nitrogen was used as nebulizing gas at a pressure of 65 psi and the flow was adjusted at 11 L/min. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full scan mass spectra of the phenolic compounds were measured from *m/z* 100 up to *m/z* 2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 100%. Mass spectrometry data were acquired in the negative ionization mode.

**HPLC-DAD System for Quantitative Analysis.** Separation of the phenolics was achieved as reported previously (*3*−*6*), with an analytical HPLC unit (Gilson), using a Spherisorb ODS2 (25.0 × 0.46 cm; 5-$\mu$m particle size) column. Detection was achieved with a Gilson DAD. Spectral data from all peaks were accumulated in the range 200−400 nm, and chromatograms were recorded at 350 nm. The data were processed on Unipointsystem software (Gilson Medical Electronics, Villiers le Bel, France).

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3- and 4-*O*-Caffeoylquinic and 3,5-dicaffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid. The other compounds were quantified as themselves.

## RESULTS AND DISCUSSION

**Qualitative Analysis.** Phenolic compounds were identified by comparison of their retention times, UV−Vis spectra in the 200−400 nm range, and MS with those obtained from standards. The identity of 3,5-dicaffeoylquinic acid was confirmed by comparison of its UV spectra and MS and chromatographic behavior with those obtained from the same compound identified in *Cynara cardunculus* (*12*).

All quince pulps presented the same chemical profile, composed by at least five identified phenolic compounds: 3-, 4-, and 5-*O*-caffeoylquinic acids, 3,5-dicaffeoylquinic acid, and rutin (**Figure 1**). The compounds previously identified in pulps were also present in peels. In this last matrix, the DAD signal data recorded at 350 nm can differentiate two groups of peaks between 39 and 62 min. The first group (39−46 min) was formed by five major peaks (peaks 5 to 9) and the second group (51−56 min) was formed by three peaks (**Figure 2**). All of the

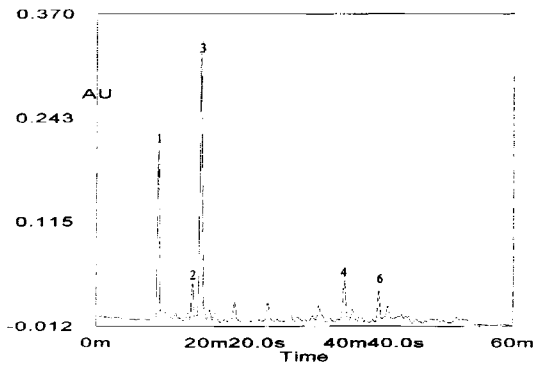


**Figure 1.** HPLC phenolic profile of a quince pulp. Detection at 350 nm. Peaks: (1) 3-*O*-caffeoylquinic acid; (2) 4-*O*-caffeoylquinic acid; (3) 5-*O*-caffeoylquinic acids; (4) 3,5-dicaffeoylquinic acid, and (6) rutin.

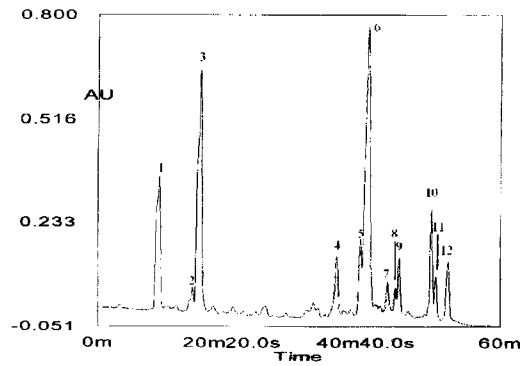


**Figure 2.** HPLC phenolic profile of a quince peel. Detection at 350 nm. Peaks: (1) 3-*O*-caffeoylquinic acid; (2) 4-*O*-caffeoylquinic acid; (3) 5-*O*-caffeoylquinic acids; (4) 3,5-dicaffeoylquinic acid; (5) quercetin 3-galactoside; (6) rutin; (7) kaempferol glycoside; (8) kaempferol 3-glucoside; (9) kaempferol 3-rutinoside; (10) and (11) quercetin glycosides acylated with *p*-coumaric acid, and (12) kaempferol glycoside acylated with *p*-coumaric acid.

peaks showed identical spectral profile, with two maxima at 257 and 353−355 nm, which indicated that they could be flavonols or flavonol derivatives. HPLC-MS analyses provided interesting information on the two mentioned groups of flavonoids. Pseudomolecular ions $[M - H]^-$ at *m/z* 463.8 and 609.5 were found for the peaks 5 and 6, respectively (**Figure 2**). Fragmentation of these ions provided a characteristic *m/z* at 300.9, typical mass in the negative mode of the quercetin aglycon. Injection of authentic standards of quercetin 3-galactoside and quercetin 3-rutinoside confirmed the occurrence of these compounds in the quince peel extracts. These results were in agreement with those of previous studies, which described these flavonols in quince puree (*3*), jam (*4*, *5*), and jelly (*6*). Peak 8 (**Figure 2**) had a pseudomolecular ion $[M - H]^-$ at *m/z* 448. MS of 448 event yielded main ion at *m/z* at 284.6, characteristic of kaempferol, which suggests the presence of a hexosyl kaempferol. The injection of authentic standard of kaempferol 3-glucoside confirmed the presence of this compound in the peels. Peaks 7 and 9 (**Figure 2**) had the same $[M - H]^-$ at *m/z* 593.8, and the event yielded a main ion at *m/z* 285.6 characteristic of kaempferol and minor fragments at *m/z* 448 corresponding to the loss of rhamnose (*m/z* 146) from the molecular ion. Authentic kaempferol 3-*O*-rutinoside was injected alone and with the peel quince extract. Retention time, and UV and MS profiles were the same as those for peak 9 (**Figure 2**).

**Table 1.** Recoveries of 5-*O*-Caffeoylquinic Acid, Rutin, and Kaempferol 3-Rutinoside from Spiked Pulp and Peel from Quince Fruit (quantification by external standard technique)[a]

| fruit part | phenolic compound | present (mg/kg) | added (mg/kg) | found[b] (mg/kg) | SD (mg/kg) | CV (%) | recovery (%) |
|---|---|---|---|---|---|---|---|
| pulp | 5-CQA | 79.6 | 233.2 | 284.8 | 0.99 | 0.35 | 91.0 |
| | Q-3-rut | 5.5 | 64.8 | 63.6 | 0.18 | 0.28 | 90.5 |
| | K-3-rut | ... | ... | ... | ... | ... | ... |
| peel | 5-CQA | 291.6 | 324.9 | 558.6 | 8.44 | 1.51 | 90.6 |
| | Q-3-rut | 432.7 | 216.6 | 599.3 | 2.83 | 0.47 | 92.3 |
| | K-3-rut | 36.9 | 46.9 | 84.9 | 0.66 | 0.78 | 101.3 |

[a] SD, standard deviation; CV, coefficient of variation; 5-CQA, 5-*O*-caffeoylquinic acid; Q-3-rut, rutin; K-3-rut, kaempferol 3-rutinoside. [b] mean value found for three assays for each studied concentration;

**Table 2.** Phenolic Composition of Pulps and Peels from Quince Fruit[a]

| | | phenolic compounds (mg/kg) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| fruit part | geographic origin | 3-CQA (9 m, 9 s) | 4-CQA (14 m, 12 s) | 5-CQA (15 m, 22 s) | 3,5-diCQA (36 m, 12 s) | Q-3-gal (39 m, 43 s) | Q-3-rut (41 m, 5 s) | K-3-glu (45 m, 2 s) | K-3-rut (45 m, 37 s) | Σ |
| pulp | Amarante | 32.4 (0.46) | 6.0 (0.06) | 79.6 (1.02) | 10.8 (0.08) | nd | 5.5 (0.20) | nd | nd | 134.3 |
| | Baião | 23.9 (0.62) | 4.1 (0.37) | 98.3 (0.98) | 8.5 (0.39) | nd | 7.4 (0.83) | nd | nd | 142.2 |
| | Vila Real | 22.7 (0.32) | 4.9 (0.26) | 54.7 (0.39) | 4.3 (0.11) | nd | 1.7 (0.10) | nd | nd | 88.3 |
| | Bragança | 0.9 (0.01) | 0.6 (0.01) | 5.6 (0.09) | tr | nd | 4.6 (0.08) | nd | nd | 11.7 |
| | Covilhã | 34.7 (1.10) | 5.3 (0.01) | 108.0 (0.68) | 3.7 (0.10) | nd | 4.2 (0.15) | nd | nd | 155.9 |
| | Viseu | 30.2 (0.02) | 5.0 (0.07) | 65.7 (1.04) | 3.6 (0.17) | nd | 5.2 (0.57) | nd | nd | 109.7 |
| | Pinhel | 56.3 (0.70) | 7.3 (0.05) | 185.6 (2.31) | 6.3 (0.20) | 4.2 (0.32) | 8.6 (0.06) | nd | nd | 268.3 |
| | min | 0.9 | 0.6 | 5.6 | tr | nd | 1.7 | - | - | 11.7 |
| | max | 56.3 | 7.3 | 185.6 | 10.8 | 4.2 | 8.6 | - | - | 268.3 |
| | mean | 28.7 | 4.7 | 85.4 | 5.3 | 0.6 | 5.3 | - | - | 130.1 |
| | SD | 16.56 | 2.09 | 55.45 | 3.56 | 1.59 | 2.24 | - | - | 77.58 |
| peel | Amarante | 117.2 (2.82) | 16.7 (0.72) | 291.6 (0.91) | 38.3 (1.74) | 26.1 (0.60) | 432.7 (47.62) | 16.7 (0.60) | 36.9 (0.61) | 976.2 |
| | Baião | 32.8 (1.34) | 5.7 (0.16) | 180.3 (10.10) | 16.0 (0.40) | 252.0 (8.48) | 872.5 (20.35) | 92.9 (2.64) | 140.2 (1.85) | 1592.4 |
| | Vila Real | 69.0 (1.72) | 10.9 (0.15) | 153.4 (4.69) | 15.4 (0.73) | 48.3 (1.66) | 223.2 (9.17) | 5.2 (0.14) | 13.1 (0.51) | 538.5 |
| | Bragança | 0.3 (0.02) | 0.3 (0.01) | 5.9 (0.04) | 2.1 (0.13) | 33.9 (2.62) | 172.3 (1.96) | 8.5 (0.11) | 20.2 (0.59) | 243.5 |
| | Covilhã | 11.3 (0.19) | 2.4 (0.01) | 58.3 (0.94) | 5.7 (0.81) | 138.1 (1.36) | 469.5 (7.28) | 45.6 (0.70) | 61.2 (0.52) | 792.1 |
| | Viseu | 56.6 (0.86) | 8.8 (0.30) | 134.5 (1.42) | 13.7 (0.63) | tr | 614.7 (10.76) | 37.2 (0.46) | 79.7 (1.14) | 945.2 |
| | Pinhel | 101.5 (3.30) | 20.4 (0.81) | 432.6 (17.25) | 22.8 (0.64) | 207.2 (6.10) | 836.0 (24.18) | 41.8 (0.71) | 76.3 (1.29) | 1738.6 |
| | min | 0.3 | 0.3 | 5.9 | 2.1 | tr | 172.3 | 5.2 | 13.1 | 243.5 |
| | max | 117.2 | 20.4 | 432.6 | 38.3 | 252.0 | 872.5 | 92.9 | 140.2 | 1738.6 |
| | mean | 55.5 | 9.3 | 179.5 | 16.3 | 100.8 | 517.3 | 35.4 | 61.1 | 975.2 |
| | SD | 44.01 | 7.33 | 143.85 | 11.89 | 98.78 | 274.53 | 30.11 | 43.58 | 536.07 |

[a] Values are expressed as mean (standard deviation) of three assays for each sample. Abbreviations: nd, not detected; tr, traces; Σ, sum of the determined phenolics; 3-CQA, 3-*O*-caffeoylquinic acid; 4-CQA, 4-*O*-caffeoylquinic acid; 5-CQA, 5-*O*-caffeoylquinic acid; 3,5-diCQA, 3,5-dicaffeoylquinic acid; Q-3-gal, quercetin 3-galactoside; Q-3-rut, rutin; K-3-glu, kaempferol 3-glucoside; K-3-rut, kaempferol 3-rutinoside. Retention times are given in parentheses below each phenolic compound abbreviaiton.

Therefore, peak 9 was identified as kaempferol 3-*O*-rutinoside, whereas peak 7 is probably an isomer of compound 9.

Peaks 10−12 (**Figure 2**) had UV spectra typical of acylated flavonoids with a cinnamic acid type (10 and 11, UV 256 shoulder, 266, 294 shoulder, 314, 358 shoulder nm; 12, UV 266, 296 shoulder, 314, 256 shoulder nm). Their $[M - H]^-$ ($m/z$ = 609.8 for compounds 10 and 11 and $m/z$ = 593.8 for compound 12) correspond to a quercetin + hexose + *p*-coumaric acid and a kaempferol + hexose + *p*-coumaric acid, respectively.

A quince peel extract was submitted to a saponification with NaOH 4 N, for 8 h in the presence of $N_2$, in the dark, at room temperature. The product was directly injected, after filtration through 0.45 $\mu$m, into the HPLC-DAD/MS for cinnamic acid and flavonoid glycosides analysis. As a result of this saponification we obtained caffeic acid (from caffeoylquinic acids) ($m/z$ 135, M-45) and *p*-coumaric acid (probably from the previously referenced acylated flavonoids, peaks 10−12) ($m/z$ 119, M-45), hexosyl quercetin, rutin, hexosyl kaempferol, and rhamnohexosyl kaempferol. Meanwhile, peaks 10−12 disappeared from the chromatogram after saponification, which means that they are probably quercetin and kaempferol glycosides acylated with *p*-coumaric acid.

So, quince peels were characterized by the presence of eight identified phenolics: 3-, 4-, and 5-*O*-caffeoylquinic acids, 3,5-dicaffeoylquinic acid, quercetin 3-galactoside, rutin, kaempferol 3-glucoside, and kaempferol 3-rutinoside, and several non-fully identified compounds (probably kaempferol glycoside and quercetin and kaempferol glycosides acylated with *p*-coumaric acid) (**Figure 2**).

As pear and apple also belong to the *Rosaceae* family, it is important to compare the phenolic profiles of these three fruits. Apple is characterized by the presence of (+)-catechin, 5-*O*-caffeoylquinic acid, (−)-epicatechin, *p*-coumaric acid, phloretin 2′-xylosylglucoside, phloretin 2′-glucoside, rutin, quercetin 3-xyloside, and quercetin 3-rhamnoside (*2*, 3). Pear contains 3-*O*-caffeoylquinic acid, (+)-catechin, *p*-hydroxybenzoic acid, 5-*O*-caffeoylquinic acid, (−)-epicatechin, quercetin 3-galactoside, quercetin 3-rhamnoside, and arbutin (*2*, 3). These two fruits have some compounds that can be considered their chemical markers: phloretin 2′-xylosylglucoside and phloretin 2′-glucoside for apple, and arbutin for pear (3−6). So, the addition of apple or pear to quince purees, jams, and jellies can be easily detected by the presence of their characteristic compounds.

From the available literature (*4*, *5*) on quince jams, we verified that in most samples the described profile (based on HPLC-

DAD analysis) was composed by 3-, 4-, and 5-*O*-caffeoylquinic acids, quercetin-3-galactoside, rutin, quercetin-3-xyloside, and quercetin-3-rhamnoside. The present work, in which MS was added to the analytical tools, makes us to think that, most probably, the compounds previously identified as quercetin 3-xyloside and quercetin-3-rhamnoside were not correctly identified, and they correspond to peaks 7 (unidentified kaempferol glycoside) and 8 (kaempferol 3-glucoside) from **Figure 2** (phenolic profile of a quince peel). Some of the compounds present in jams are characteristic of quince peel, which suggests that the manufacturers use all of the quince fruit, and not only the pulp as recommended by Portuguese Legislation (*1*).

**Quantitative Analysis.** Recently an HPLC-DAD method was developed for determination of the phenolic profile in quince jams (*5*). Once we were dealing with similar matrixes, this technique was applied to quince pulps and peels. Given the similarity of the chemical structures between the several caffeoylquinic acids, quercetin, and kaempferol glycosides, and, therefore, their UV spectra and absorptivity, the recoveries of the extractive method were determined only for 5-*O*-caffeoylquinic acid and rutin in quince pulp and peel, and kaempferol 3-rutinoside in peel. So, to test the recovery of the procedure, one quince pulp and one peel were added to known quantities of each one of the reference phenolics. The samples were analyzed in triplicate before and after the additions. Recovery values were high (between 90.5 and 101.3%) (**Table 1**). This procedure demonstrated the effectiveness of the extraction and the accuracy of the method.

In quince pulps, the most abundant phenolic was 5-*O*-caffeoylquinic acid. Generally, except for the pulp from Bragança, the second major compound was 3-*O*-caffeoylquinic acid (**Table 2**).

The most abundant compound in quince peels was either 5-*O*-caffeoylquinic acid or different flavonol derivatives according to geographic origin. In all cases, quince peel had a higher amount of phenolics than was found in quince pulp (**Table 2**).

In conclusion, this study suggests that the analysis of the phenolic compounds in quince fruit is quite useful in the characterization of its pulp and peel, allowing the discrimination of these two parts of the fruit. Quince pulp has a much lower amount of phenolics than quince peel. Besides, quince pulp and peel differ distinctly in their phenolic profiles: the pulp contains mainly caffeoylquinic acids, whereas the peel possesses both caffeoylquinic acids and several flavonol glycosides, in great amounts.

This procedure also allows the detection of adulterations in quince jams by addition of quince peel.

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# Approach to the Study of *C*-Glycosyl Flavones by Ion Trap HPLC-PAD-ESI/MS/MS: Application to Seeds of Quince (*Cydonia oblonga*)

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**Ion trap HPLC-PAD-ESI/MS/MS has been used to study *C*-glycosyl flavones in quince seeds. Comparative analysis of the ions $[(M-H)-60]^-$, $[(M-H)-90]^-$ and $[(M-H)-120]^-$ from 6-*C*- and 8-*C*-glycosyl flavone isomers, together with their respective retention times, allowed deductions to be made about the nature of the sugar units and the positions of *C*-glycosylation. Vicenin-2 (6,8-di-*C*-glucosyl apigenin), lucenin-2 (6,8-di-*C*-glucosyl luteolin), stellarin-2 (6,8-di-*C*-glucosyl chrysoeriol), isoschaftoside (6-*C*-arabinosyl-8-*C*-glucosyl apigenin), schaftoside (6-*C*-glucosyl-8-*C*-arabinosyl apigenin), 6-*C*-pentosyl-8-*C*-glucosyl chrysoeriol and 6-*C*-glucosyl-8-*C*-pentosyl chrysoeriol were identified in quince seed.** **Copyright © 2003 John Wiley & Sons, Ltd.**

*Keywords:* HPLC-PAD-MS/MS; electrospray ionisation; ion trap; *C*-glycosyl flavones; *Cydonia oblonga* Miller; quince.

## INTRODUCTION

As part of a continuing study concerning the analysis of phenolic compounds from quince fruit (Andrade *et al.*, 1998; Silva *et al.*, 2002 and references therein), we have been interested in screening the flavonoids in quince seed. From a preliminary examination of these compounds by reversed-phase HPLC, UV spectra and resistance to acidic hydrolysis, it was deduced that a number of *C*-glycosyl flavones were present. However, the analysis of this class of compound is not only difficult but was exacerbated in the present case due to the scarcity of plant material.

The use of MS in the analysis of flavonoids and their glycosides has been recently reviewed (Stobiecki, 2000). When this technique was first applied to the study of the non-volatile and thermally labile glycosyl flavonoids, a derivatisation procedure was necessary prior to analysis by electron impact (EI)/MS. In such cases permethylation (or per-deuteromethylation) was employed in order to avoid excessive increases in the masses of the analytes. For the *C*-glycosyl flavones, examples of the use of this strategy for the structural determination (i.e. identification of sugar units and positions of glycosylation) of mono-*C*-, di-*C*- and *O*-glycosyl-*C*-glycosyl flavones have been reported by Bouillant *et al.* (1979, 1984 and references contained therein). Later, the use of fast atom bombardment (FAB)/MS and field desorption/MS permitted the study of such compounds without the need for derivatisation, and it was reported (Chopin and Dellamonica, 1988) that the conclusions obtained by these techniques were in good agreement with those obtained by EI/MS of the permethylated derivatives. Becchi and Fraisse (1989) used collision-induced dissociation (CID)/mass analysed ion kinetic energy (MIKE) for the study of mono-*C*- and di-*C*-glycosyl flavones, whilst Li *et al.* (1991, 1992) and Claeys *et al.* (1996) also employed CID/FAB for the differentiation of *C*-glycosyl flavone isomers. Bakhtiar *et al.* (1994) studied 17 *C*-glycosyl flavones (mono-*C*-glycosyl flavones, di-*C*-glycosyl flavones, *C*-glycosyl flavone-2″-*O*-glycosides and di-*C*-glycosyl flavone-2″-*O*-glycosides) by desorption chemical ionisation (DCI)/MS, which enabled the characterisation of the aglycone, the sugar sequence, the nature of the *C*-sugar (hexose, pentose) etc., and also provided information about the different ions produced. Generally, with any of these techniques, the preferential fragmentation of *C*-glycosyl flavones was that of the sugar in position 6 and, from a comparative study of the relative abundance of the obtained fragments in both isomers, it was possible to draw conclusions about the sugar type and the position of *C*-glycosylation.

The use of HPLC coupled with MS is extremely valuable as a screening tool where the scarcity of source material prevents the ready isolation and purification of compounds for later identification, and especially for the analysis of difficult metabolites such as the *C*-glycosyl flavones (for a review see Careri *et al.*, 1998). Grayer *et al.* (2000) used atmospheric pressure chemical ionisation (APCI)/MS coupled with HPLC for the study of *C*-glycosyl flavones, whilst Waridel *et al.* (2001) differentiated *C*-glycoside isomers using HPLC-CID/MS under various energy conditions.

In the present paper, we describe an application of ion trap HPLC–photodiode array detection (PAD)–electrospray ionisation (ESI)/MS/MS to determine the structures of *C*-glycosyl flavones in methanolic extracts of quince seeds (*Cydonia oblonga* Miller).

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## EXPERIMENTAL

**Plant materials and extraction of phenolic compounds.** Studies were carried out on seeds from healthy and ripe quince fruits, harvested during September/October 2000 from different locations in northern and central Portugal. Seeds were freeze-dried using a Labconco (Kansas City, MO, USA) model 4.5 apparatus and, owing to the scarcity of plant material, equal portions of lyophilised seeds from each geographical region were combined and pulverized. The powdered sample (ca. 1.5 *g*) was exhaustively extracted with methanol (i.e. until the residue gave a negative reaction for phenolic compounds with 20% sodium hydroxide). The extract was then filtered, evaporated to dryness under reduced pressure at 40°C and redissolved in methanol (1 mL).

**Flavonoid standards.** The *C*-glycosyl flavones used as standards for co-chromatography during the HPLC analysis were from the collection of the Laboratorio de Fitoquímica del Departamento de Ciencia y Tecnología de los Alimentos, Murcia, Spain. The acid hydrolysis of the *O*-glycosyl-*C*-glycosyl flavones, used as standards, was carried out by dissolving a small sample (<1 mg) in 4 M hydrochloric acid (1 mL) in a tube which was then sealed and maintained for 3 h in an oven at 90°C. Under these conditions the asymmetric 5-hydroxy-*C*-glycosyl flavones were subjected to isomerisation, resulting from a Wessely–Moser rearrangement, and a mixture of the two isomers was thus produced. The resulting mixture was membrane (0.45 μm) filtered and directly analysed by HPLC-PAD-ESI/MS/MS.

**HPLC-PAD-MS analysis.** The HPLC system employed consisted of an Agilent (Waldbronn, Germany) model G1312A binary pump, a G1313 A autosampler, and a G1322A degasser coupled with a G1315B PAD (controlled by A.08.03 software) and an Agilent 1100 series LC/MSD trap connected in series. The mass detector was an Agilent G2445A ion-trap MS equipped with an ESI system (controlled by 4.0.25 software). Nitrogen was used as the nebulizing gas at a pressure of 65 psi and a flow rate of 11 mL/min. The heated capillary and the voltage were maintained at 350°C and 4 kV, respectively. Full scan MS of the phenolic compounds were measured from *m/z* 100 to 1000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas. The collision energy was set at 100%. MS data were acquired in the negative ionisation mode. MS/MS data were acquired in the automatic mode.

Chromatographic separations were carried out on a LiChrocart RP-18 (Merck, Darmstadt, Germany) column (250 × 4 mm i.d.; 5 μm particle size) and guard column (4 × 4 mm i.d.). The mobile phase comprised two solvents, namely, water:formic acid (95:5, v/v; solvent A) and methanol (solvent B), in a gradient program which was initially 95:5 (A:B) and changed to 85:15 at 3 min, 75:25 at 13 min, 70:30 at 25 min, 45:55 at 35 min, 45:55 at 45 min, 20:80 at 46 min, 20:80 at 50 min, 95:5 at 52 min, and finally 95:5 at 60 min. The flow rate was 0.9 mL/min, and the sample injection volumes were variable between 20 and 80 μL. The chromatograms were recorded at 270 nm and spectral data for all peaks were accumulated in the range 240–400 nm.

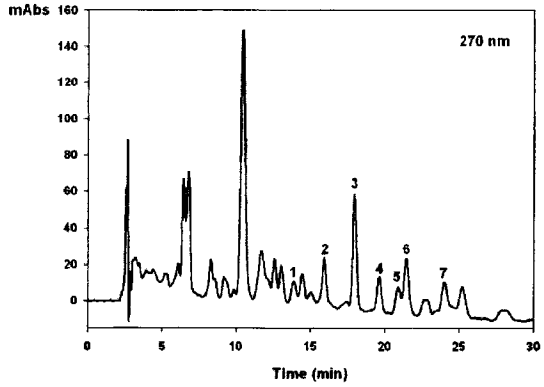


**Figure 1.** HPLC chromatogram of a methanol extract of quince seed (detection at 270 nm). Key to peak identity: **1**, 6,8-di-*C*-glucosyl luteolin; **2**, 6,8-di-*C*-glucosyl apigenin; **3**, 6,8-di-*C*-glucosyl chrysoeriol; **4**, 6-*C*-arabinosyl-8-*C*-glucosyl apigenin; **5**, 6-*C*-glucosyl-8-*C*-arabinosyl apigenin; **6**, 6-*C*-pentosyl-8-*C*-glucosyl chrysoeriol; **7**, 6-*C*-glucosyl-8-*C*-pentosyl chrysoeriol. (For chromatographic protocol see Experimental section.)

## RESULTS AND DISCUSSION

The HPLC-PAD chromatogram of quince seed extract (Fig. 1) showed mobilities of flavonoids which were characteristic of glycoside derivatives. Acid hydrolysis (see Experimental section) did not, however, modify the retention times of these compounds indicating that they were *C*-glycosyl flavones. The MS of the analytes did not exhibit molecular ion fragmentation reinforcing this suggestion.

Analysis of the UV spectra (Mabry *et al.*, 1970) of the analytes revealed that there were two groups of flavonoids present, a group with one substituent in ring B (compounds **2**, **4** and **5**), and another with two substituents in ring B (compounds **1**, **3**, **6** and **7**). It appeared that in each case positions 6 and/or 8 were substituted since the maximum of band II was at 270 nm or higher.

### Flavonoids with one substituent in the B ring

**Compound 2.** Retention time, 15.9 min; UV, 259 (sh), 271, 335 nm; MS, 593 $[M-H]^-$; MS/MS $[M-H]^-$, 575 $[(M-H)-18]^-$, 503 $[(M-H)-90]^-$, 473 $[(M-H)-120]^-$, 383 $[(M-H)-210]^-$ (A [270, aglycone apigenin]+113), 354 $[(M-H)-239]^-$ (A+84). The losses in the MS/MS coincided with those reported by Bakhtiar *et al.* (1994) and Grayer *et al.* (2000) for di-*C*-hexosyl flavones and suggested the presence of apigenin (270) + hexose (162) + hexose (162) (molecular weight 594). The retention time, UV spectra and MS/MS of vicenin-2 [from *Spergularia rubra* (Bouillant *et al.*, 1979)] when co-chromatographed with **2** coincided, thus confirming the identity of the latter as 6,8-di-*C*-glucosyl apigenin (vicenin-2).

**Compound 4.** Retention time, 19.9 min; UV, 259 (sh), 272, 333 nm; MS, 563 $[M-H]^-$; MS/MS $[M-H]^-$, 545 $[(M-H)-18]^-$, 503 $[(M-H)-60]^-$, 473 $[(M-H)-90]^-$, 443 $[(M-H)-120]^-$, 383 $[(M-H)-180]^-$ (A [270, aglycone

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apigenin]+113), 354 [(M-H)-209]$^-$ (A+84). These results are in agreement with those reported by Bakhtiar *et al.* (1994) for di-*C*-hexosyl-pentosyl flavones (probably with glucose since it is the most usual hexose) of the form: apigenin (270) + glucose (162) + pentose (132) (molecular weight 564). Hence **4** was concluded to be 6-*C*-glucosyl-8-*C*-pentosyl apigenin or 6-*C*-pentosyl-8-*C*-glucosyl apigenin.

**Compound 5.** Retention time, 21.2 min; UV, 259 (sh), 270, 335 nm; MS, 563 [M-H]$^-$; MS/MS [M-H]$^-$, 545 [(M-H)-18]$^-$, 503 [(M-H)-60]$^-$, 473 [(M-H)-90]$^-$, 443 [(M-H)-120]$^-$, 383 [(M-H)-180]$^-$ (A [270, aglycone apigenin]+113), 354 [(M-H)-209]$^-$ (A+84). The data suggested that **5** was an isomer of **4**, namely, 6-*C*-pentosyl-8-*C*-glucosyl apigenin or 6-*C*-glucosyl-8-*C*-pentosyl apigenin.

In previous studies using other MS techniques (mainly EI, FAB, DCI, and APCI), the preferential fragmentation of *C*-glycosyl flavones was that of the sugar in position 6 relative to the sugar in position 8, and with ESI the behaviour should be similar. On comparing the fragmentation of **2** with those of **4** and **5**, it was observed that **2** did not show the ion [(M-H)-60]$^-$ at *m/z* 503 which was present in the spectra of the other two compounds. This ion must, therefore, have had its origin in the pentose. This ion was much more abundant in **4** than in **5** (Table 1), which led to the suggestion that **4** was the isomer with pentose in position 6. In compounds **2** and **5**, with a hexose in position 6, the base peak was [(M-H)-120]$^-$.

In order to obtain more information about **4** and **5**, HPLC-PAD-ESI/MS/MS analysis of two *C*-pentosyl-*C*-hexosyl apigenin isomers, namely, isoschaftoside (6-*C*-arabinosyl-8-*C*-glucosyl apigenin) and schaftoside (6-*C*-glucosyl-8-*C*-arabinosyl apigenin) produced by the acid hydrolysis of 6″-*O*-glucosyl-schaftoside from *Stellaria holostea* (Bouillant *et al.*, 1984), was carried out. Under the conditions of acid hydrolysis indicated in the Experimental section, the 5-hydroxy-*C*-glycosylflavones were subjected to isomerisation resulting from a Wessely–Moser rearrangement, and this gave rise to a mixture of two isomers. Comparison of retention times, UV spectra and MS/MS of these isomers when co-chromatographed with **4** and **5** confirmed the structures of the latter as 6-*C*-arabinosyl-8-*C*-glucosyl apigenin and 6-*C*-glucosyl-8-*C*-arabinosyl apigenin, respectively.

Compound **2** showed important losses from the pseudomolecular ion [M-H]$^-$ of 90 and 120 (base peak), whilst the pseudomolecular ion of **4** and isoschaftoside presented losses of 18, 60, 90 and 120, all of them very abundant (with [(M-H)-90]$^-$ being the base peak). In contrast, in **5** and schaftoside (compounds with glucosylation at position 6), the [(M-H)-60]$^-$ ion, characteristic of pentose derivatives, was small because this sugar was in position 8 and fragmentation in this position was not preferential. Similarly, the [(M-H)-18]$^-$ ion was more abundant when pentose was in position 6, and therefore it showed low abundance in **2**, **5** and schaftoside (Table 1).

Other important ions that could be observed in the MS of these three compounds were those due to the aglycone plus the residues of the sugars that remained linked to it, i.e. A+84 and A+113. These ions indicated the substitution (hydroxyl/methoxyl groups) of the aglycone. In the case of a di-*C*-hexosyl flavone, A+84 and A+113 were from the loss of 239 and 210 from the pseudomolecular ion, respectively. In the case of a di-*C*-substituted flavone

**Table 1. Retention times of selected *C*-glycosyl flavones and relative abundances of ions obtained from the respective pseudomolecular ions [M-H]$^-$ by MS/MS**

| Compounds[a] | Retention time (min) | Relative abundances (%) of ions from [M-H]$^-$ | | | | | |
|---|---|---|---|---|---|---|---|
| | | –18 | –60 | –90 | –120 | A+113 | A+83 |
| *6,8-di-C-symmetricglycosyl flavones* | | | | | | | |
| **1** (6,8-di-*C*-glc luteolin) | **13.8** | 3 | — | 22 | 100 | 32 | 39 |
| 6,8-di-*C*-glc luteolin | **13.8** | 2 | — | 16 | 100 | 3 | 5 |
| **2** (6,8-di-*C*-glc apigenin) | **15.9** | 4 | — | 27 | 100 | 42 | 45 |
| 6,8-di-*C*-glc apigenin | **15.9** | 6 | — | 30 | 100 | 17 | 20 |
| **3** (6,8-di-*C*-glc chrysoeriol) | **18.1** | 3 | — | 13 | 100 | 21 | 45 |
| 6,8-di-*C*-glc chrysoeriol | **18.1** | 3 | — | 12 | 100 | 27 | 60 |
| *6,8-di-C-asymmetricglycosyl flavones* | | | | | | | |
| **4** (6-*C*-arab-8-*C*-glc apigenin) | 19.9 | 21 | 71 | 100 | 63 | 87 | 35 |
| 6-*C*-arab-8-*C*-glc apigenin | **19.9** | 24 | 76 | 100 | 60 | 75 | 26 |
| **5** (6-*C*-glc-8-*C*-arab apigenin) | **21.2** | 6 | 6 | 69 | 100 | 53 | 65 |
| 6-*C*-glc-8-*C*-arab apigenin | 21.2 | 6 | 8 | 59 | 100 | 47 | 41 |
| **6** (6-*C*-pent-8-*C*-glc chrysoeriol) | 21.8 | 9 | 20 | 25 | 100 | 79 | 36 |
| **7** (6-*C*-glc-8-*C*-pent chrysoeriol) | 24.0 | 9 | 6 | 94 | 100 | 55 | 73 |
| *6- and 8-mono-C-glycosyl flavones* | | | | | | | |
| 8-*C*-glc luteolin | **20.3** | — | — | 50 | 100 | — | — |
| 6-*C*-glc luteolin | **22.8** | 15 | — | 100 | 25 | — | — |
| 8-*C*-glc apigenin | **23.4** | — | — | 5 | 100 | — | — |
| 6-*C*-glc apigenin | **28.9** | 4 | — | 46 | 100 | — | — |
| 8-*C*-arab apigenin | **32.1** | — | 29 | 100 | — | — | — |
| 6-*C*-arab apigenin | **34.7** | 10 | 100 | 12 | — | — | — |
| 8-*C*-glc acacetin | **33.9** | — | — | 1 | 100 | — | — |
| 6-*C*-glc acacetin | 37.4 | — | — | 19 | 100 | — | — |

[a] glc, glucosyl; pent, pentosyl; arab, arabinosyl.

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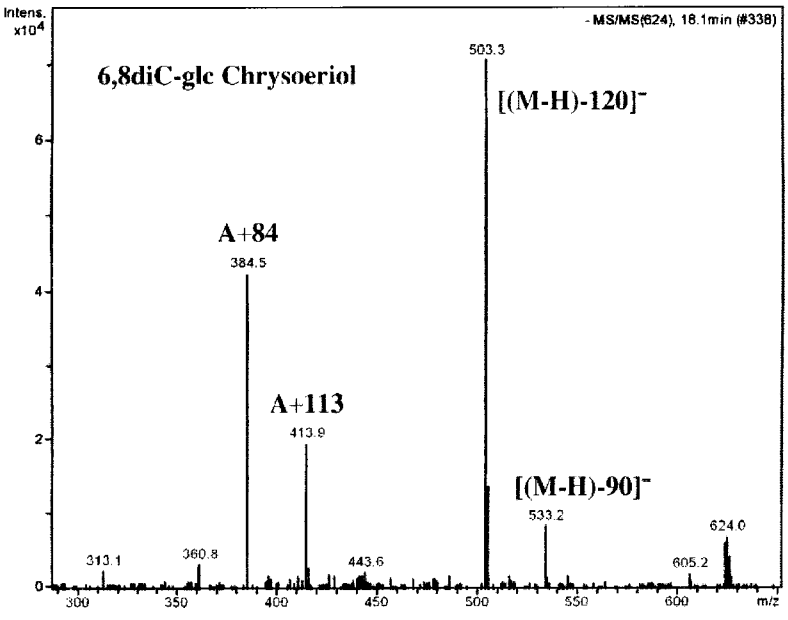


**Figure 2.** MS/MS of the pseudomolecular ion $[M-H]^-$ of the 6,8-di-*C*-symmetric glycosyl flavone, 6,8-di-*C*-glucosyl chrysoeriol.

with a hexose and a pentose, A+84 and A+113 were from the loss of 209 and 180 from the pseudomolecular ion, respectively.

**Flavonoids with two substituents in the B ring**

Four compounds present in the extract (Fig. 1), namely, **1** (molecular weight 610), **3** (624), **6** (594) and **7** (594), could be derivatives of luteolin (5,7,3′,4′-tetrahydroxyflavone; 286), chrysoeriol (5,7,4′-trihydroxy-3′-methoxyflavone; 300) or diosmetin (5,7,3′-trihydroxy-4′-methoxyflavone; 300) with hexosyl (162), rhamnosyl (146) and pentosyl (132) sugars.

**Compound 1.** Retention time, 13.8 min; UV, 258 (sh), 270, 343 nm; MS, 609 $[M-H]^-$; MS/MS, $[M-H]^-$: 591 [(M-H)-18], 519 [(M-H)-90], 489 [(M-H)-120], 399 [(M-H)-210] (A [286]+113), 369 [(M-H)-240]⁻ (A+83). The ions at *m/z* 369 (A+83) and 399 (A+113) indicated that the aglycone was luteolin (5,7,3′,4′-tetrahydroxyflavone; 286) which implied that the *C*-glycosylation must be di-*C*-hexosyl. The retention time, UV spectra and MS/MS of lucenin-2 [from *Spergularia rubra* (Bouillant *et al.*, 1979)] when co-chromatographed with **1** coincided, thus confirming its identity as 6,8-di-*C*-glucosyl luteolin (lucenin-2).

**Compound 3.** Retention time, 18.1 min; UV, 257 (sh), 270, 346 nm; MS, 623 $[M-H]^-$; MS/MS $[M-H]^-$, 605 [(M-H)-18]⁻, 533 [(M-H)-90]⁻, 503 [(M-H)-120]⁻, 413 [(M-H)-210]⁻ (A [300]+113), 384 [(M-H)-239]⁻ (A+84). These data suggested the presence of chrysoeriol or diosmetin (300) + hexose (162) + hexose (162). The retention time, UV spectra and MS/MS of stellarin-2 [from *Spergularia rubra* (Bouillant *et al.*, 1979)] when co-chromatographed with **3** coincided, thus confirming its identity as 6,8-di-*C*-glucosyl chrysoeriol (stellarin-2).

The MS/MS of **2**, **1** and **3** showed the same type of fragmentation ([(M-H)-18]⁻, [(M-H)-90]⁻, [(M-H)-120]⁻, A+113 and A+83/84; Fig. 2), indicating that the three compounds had the same glycosylation, the *m/z* values of the ions being different only because they originated from different aglycones (A), i.e. apigenin, luteolin and chrysoeriol/diosmetin, respectively. The relative abundances, namely, [(M-H)-18]⁻ < [(M-H)-90]⁻ < [(M-H)-120]⁻ (the latter being the base peak) were also very similar both in quince seed extracts and in the standards (Table 1), but for ions A+113 and A+83/84 no such relation was observed. Further, as with **2**, the [(M-H)-60]⁻ ion was not observed in the MS of **1** and **3**.

**Compound 6.** Retention time, 21.8 min; UV, 259 (sh), 270, 345 nm; MS, 593 $[M-H]^-$; MS/MS $[M-H]^-$, 575 [(M-H)-18], 533 [(M-H)-60], 503 [(M-H)-90], 473 [(M-H)-120], 413 [(M-H)-180] (A [300] + 113), 384 [(M-H)-209]⁻ (A+84). These data suggested the presence of chrysoeriol or diosmetin (300) + hexose (162) + pentose (132) indicating that **6** was either 6-*C*-hexosyl-8-*C*-pentosyl chrysoeriol/diosmetin or 6-*C*-pentosyl-8-*C*-hexosyl chrysoeriol/diosmetin.

**Compound 7.** Retention time, 24.0 min; UV, 257 (sh), 271, 347 nm; MS, 593 $[M-H]^-$; MS/MS $[M-H]^-$, 575 [(M-H)-18]⁻, 533 [(M-H)-60]⁻, 503 [(M-H)-90]⁻, 473 [(M-H)-120]⁻, 413 [(M-H)-180]⁻ (A [300] + 113), 384 [(M-H)-209]⁻ (A+84). These data clearly indicated that **7** was an isomer of **6** being either 6-*C*-pentosyl-8-*C*-hexosyl chrysoeriol/diosmetin or 6-*C*-hexosyl-8-*C*-pentosyl chrysoeriol/diosmetin.

MS/MS of the pseudomolecular ions of both **6** and **7** exhibited the following fragments: [(M-H)-18]⁻ (575), [(M-H)-60]⁻ (533), [(M-H)-90]⁻ (503) and [(M-H)-120]⁻ (473), as expected for the isomers; nevertheless, there were some differences in their intensities (Table 1). The ion [(M-H)-60]⁻ (533), that was not observed in **3**

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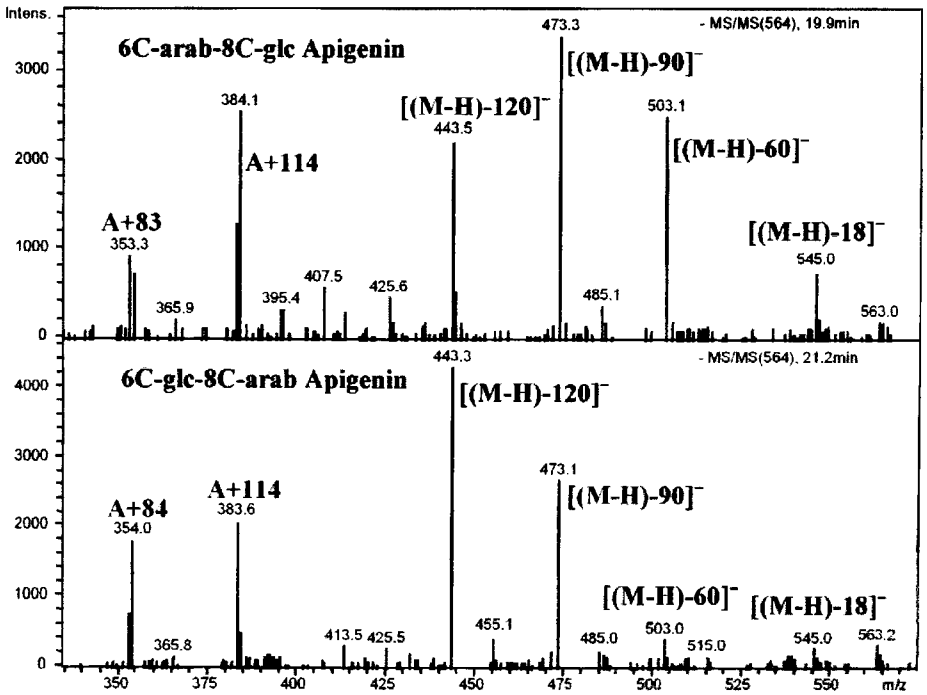


**Figure 3.** MS/MS of the pseudomolecular ions [M-H]⁻ of the 6,8-di-*C*-asymmetric glycosyl flavones, 6-*C*-arabinosyl-8-*C*-glucosyl apigenin and 6-*C*-glucosyl-8-*C*-arabinosyl apigenin.

(indicating that this ion was not from a hexose but from a pentose), was more intense in **6** than in **7**. According to reasoning already discussed, **6** must be 6-*C*-pentosyl-8-*C*-hexosyl chrysoeriol/diosmetin while **7** must be 6-*C*-hexosyl-8-pentosyl-*C*-pentosyl chrysoeriol/diosmetin.

Compounds **6** and **7** have MS/MS which are differentiated by the higher abundance of ion $[(M-H)-60]^-$ in **6** (6-*C*-pentosyl) relative to **7** (6-*C*-glucosyl) (Fig. 3) confirming that the abundance of this ion is a marker for the 6-*C*-pentosylation. The relative abundances in the order $[(M-H)-60]^- < [(M-H)-90]^- < [(M-H)-120]^-$ in **6** compared with $[(M-H)-120] < [(M-H)-60] < [(M-H)-90]$ in **4** and isoschaftoside (Table 1) suggested that the pentose present in **6** was not the same as that present in **4** and isoschaftoside. The presence of stellarin-2 as the main flavonoid in this matrix, plus the existence of glucose as the only hexose detected in the flavonoids from quince seed, led us to postulate that the most probable structures for **6** and **7** are 6-*C*-pentosyl-8-*C*-glucosyl chrysoeriol and 6-*C*-glucosyl-8-*C*-pentosyl chrysoeriol, respectively.

In order to confirm these results and to gain more general knowledge about the MS/MS fragmentation of these types of compounds, several mono-*C*-glycosyl flavones were analysed under the same conditions.

**Vitexin (8-*C*-glucosyl apigenin).** Vitexin resulted from acid hydrolysis of 2″-*O*-xylosyl vitexin from *Beta vulgaris* (Gil *et al.*, 1998). Retention time, 23.4 min; MS, 431 $[M-H]^-$; MS/MS $[M-H]^-$, 341 $[(M-H)-90]^-$, 311 $[(M-H)-120]^-$.

**Isovitexin (6-*C*-glucosyl apigenin).** Isovitexin resulted from the acid treatment of 2″-*O*-xylosyl vitexin (Wessely–Moser acid isomerisation). Retention time, 28.9 min; MS, 431 $[M-H]^-$; MS/MS $[M-H]^-$, 413 $[(M-H)-18]^-$, 341 $[(M-H)-90]^-$, 311 $[(M-H)-120]^-$. The MS/MS of the pseudomolecular ion of these two isomers could be differentiated by the larger abundance of the ion at *m/z* 341 $[(M-H)-90]^-$ in isovitexin relative to vitexin (Table 1): isovitexin also exhibited the ion $[(M-H)-18]^-$, although in small amounts.

**Cytisoside (8-*C*-glucosyl acacetin).** Cytisoside was kindly supplied by Professor Chopin (University of Lyon, France). Retention time 33.9 min: MS, 445 $[M-H]^-$; MS/MS $[M-H]^-$, 355 $[(M-H)-90]^-$, 325 $[(M-H)-120]^-$.

**Isocytisoside (6-*C*-glucosyl acacetin).** Isocytisoside was obtained by acid isomerisation of cytisoside. Retention time 37.4 min; MS, 445 $[M-H]^-$; MS/MS $[M-H]^-$, 355 $[(M-H)-90]^-$, 325 $[(M-H)-120]^-$. In this pair of isomers the ion at *m/z* 355 $[(M-H)-90]^-$ was present in the 6-*C*-glucosyl derivative with an abundance of 19% whilst in the 8-*C*-glucosyl derivative it appeared only in traces (1%).

**Orientin (8-*C*-glucosyl luteolin).** Orientin was obtained by acid hydrolysis of 2″-*O*-glucosyl orientin from *Stenotaphrum secundatum* (Ferreres *et al.*, 1984). Retention time 20.3 min; MS, 447 $[M-H]^-$; MS/MS $[M-H]^-$, 357 $[(M-H)-90]^-$, 327 $[(M-H)-120]^-$.

**Isoorientin (6-*C*-glucosyl luteolin).** Isoorientin was obtained by acid isomerisation of orientin. Retention time 22.8 min; MS, 447 $[M-H]^-$; MS/MS $[M-H]^-$, 429 $[(M-H)-18]^-$, 357 $[(M-H)-90]^-$, 328 $[(M-H)-119]^-$. As with the previous compounds, the ion at $[(M-H)-90]^-$ which

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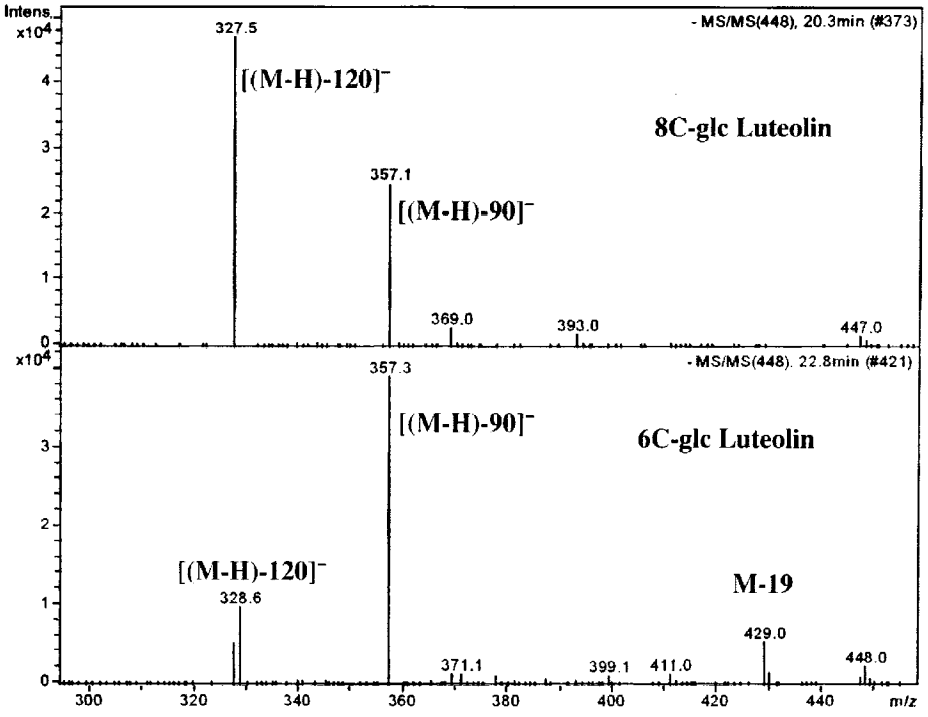


**Figure 4.** MS/MS of the pseudomolecular ions $[M-H]^-$ of the 6- and 8-mono-*C*-hexosyl flavones, 6-*C*-glucosyl luteolin and 8-*C*-glucosyl luteolin.

characterises the 6-*C*-glucosylation was more abundant in isoorientin, being the base peak in this case. The $[(M-H)-18]^-$ ion was absent from the MS/MS of orientin.

**Isomollupentin (6-*C*-arabinosyl apigenin).** Isomollupentin was obtained by acid hydrolysis of 7, 2″-di-*O*-glucosyl-6-*C*-arabinosyl apigenin from *Spergularia rubra* (Bouillant *et al.*, 1979). Retention time 34.7 min; MS, 401 $[M-H]^-$, 383 $[M-19]^-$, 342 $[M-60]^-$, 312 $[M-90]^-$; MS/MS $[M-H]^-$, 383 $[(M-H)-18]^-$, 341 $[(M-H)-60]^-$, 312 $[(M-H)-90]^-$.

**Mollupentin (8-*C*-arabinosyl apigenin).** Mollupentin was obtained by acid isomerisation of isomollupentin. Retention time 32.1 min; MS, 401 $[M-H]^-$; MS/MS $[M-H]^-$, 341 $[(M-H)-60]^-$, 311 $[(M-H)-90]^-$. The ion at $[(M-H)-60]^-$ (which characterises the presence of a pentose) was present in both of these isomers, as was the case for the 6-*C*-pentosyl-8-*C*-hexosylflavones, being the base peak in isomollupentin whereas the base peak in mollupentin was at *m/z* $[(M-H)-90]^-$. In neither of these isomers could the ion $[(M-H)-120]^-$ be observed even though this was a very important peak (the base peak in almost all cases) in the mono-*C*-glucosyl flavones and in the di-*C*-glycosyl flavones when any of the sugars present were glucose.

Although the number of examples outlined above is not large, several conclusions may be drawn from the study of the ions $[(M-H)-18]^-$, $[(M-H)-60]^-$, $[(M-H)-90]^-$, $[(M-H)-120]^-$ of the MS/MS from the analysed compounds.

In mono-*C*-glycosyl flavones the presence of $[(M-H)-120]^-$, and the simultaneous absence of $[(M-H)-60]^-$, indicated a hexose as the sugar of *C*-glycosylation; in such cases, the ion $[(M-H)-90]^-$ could also be observed, being more abundant in the 6-*C*-glucosyl derivatives (sometimes as the base peak) than in the 8-*C*-glucosyl derivatives (where it might show very low abundance). The ion $[(M-H)-18]^-$ was detected with greater frequency in 6-*C*-glucosyl derivatives than in 8-*C*-glucosyl derivatives (Fig. 4).

In the single example of a mono-*C*-pentosyl flavone, the absence of $[(M-H)-120]^-$, plus the presence of $[(M-H)-60]^-$, indicated a pentose as the sugar of *C*-glycosylation; this ion was more abundant in the 6-*C*-pentosyl derivative (being the base peak in the example studied) than in the 8-*C*-pentosyl derivative. Both isomers exhibited the ion $[(M-H)-90]^-$, which was the base peak in the 8-*C*-pentosyl derivative (Fig. 5).

In mono-*C*-glucosyl flavones, the peaks that could give clues about the nature of the aglycone (i.e. substitution with hydroxyl or methoxyl groups) were A+41 and A+71 which corresponded, respectively, to $[(M-H)-120]^-$ and $[(M-H)-90]^-$ when the sugar of *C*-glycosylation was a hexose and to $[(M-H)-90]^-$ and $[(M-H)-60]^-$ when the sugar was a pentose. Furthermore, in the HPLC system described, the 8-*C*-glycosyl derivative eluted before its *C*-6 isomer, information which could be helpful in the differentiation of the isomers.

From the MS/MS data of the di-*C*-glycosyl flavones studied in the present work, ESI (as with other MS techniques) gave rise to preferential fragmentation of the sugar linked to *C*-6 rather than that linked to *C*-8, although partial fragments of both sugars were produced leading to losses from the molecular ion. In symmetrical

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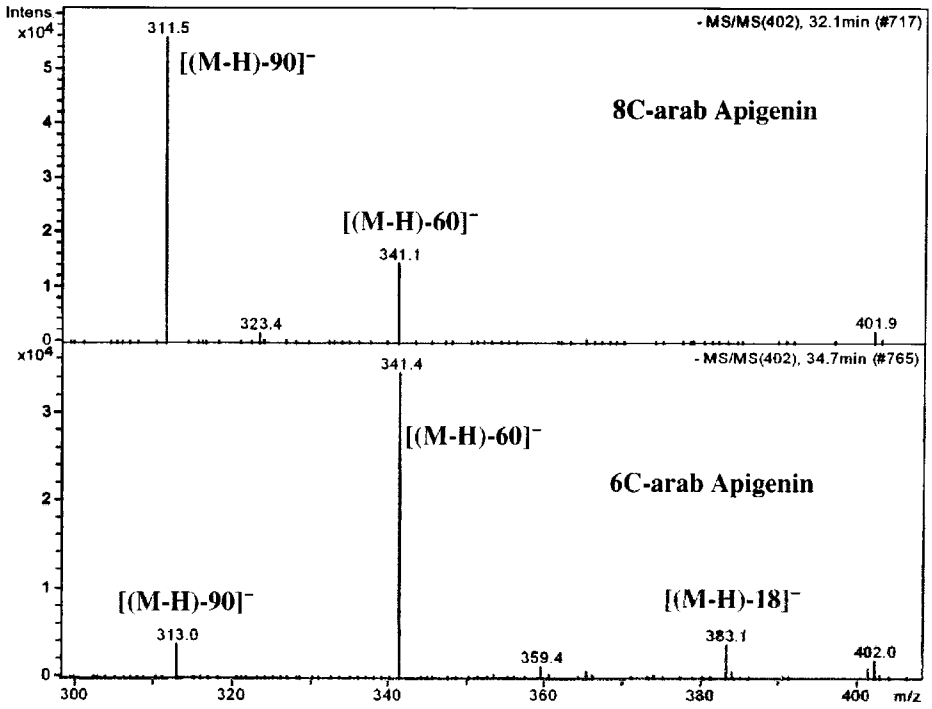


**Figure 5.** MS/MS of the pseudomolecular ions [M-H] of the 6- and 8-mono-*C*-pentosyl flavones, 6-*C*-arabinosyl apigenin and 8-*C*-arabinosyl apigenin.

di-*C*-glycosyl flavones (Fig. 2: presence of only glucose in the three studied examples) the main ions observed were [(M-H)-90] and [(M-H)-120] (base peak); in asymmetric di-*C*-glycosyl flavones (di-*C*-substituted flavonoids with different sugars, glucose and pentose) the ions $[(M-H)-60]^-$ and $[(M-H)-18]^-$ could also be observed (with $[(M-H)-18]^-$ being present, although very small, in the presence of only glucose) and these ions were more intense in the 6-*C*-derivatives than in the 8-*C*-derivatives. In all cases, the ions A+83/84 and A+113/114 could be observed which characterised the substitution of the aglycone (Fig. 3). In the HPLC system described, for asymmetric di-*C*-glycosyl flavones with glucose and pentose, the 6-*C*-pentosyl-8-*C*-glucosyl eluted before the isomeric 6-*C*-glucosyl-8-*C*-pentosyl.

In conclusion, the study of a *C*-glycosyl flavone and the establishment of the *C*-glycosylation position, requires the comparison of both the retention times and the $m/z$ of ions obtained by MS/MS of the molecular ion of the original product, with those of the product resulting from acid treatment (Wessely–Moser isomerisation) of the original analyte. In those cases where there is insufficient plant material for the isolation of these secondary metabolites for later structural study, ion trap HPLC-PAD-ESI/MS/MS provides a good screening method for C-glycosyl flavones. However, more model compounds need to be studied before it will be possible to be certain about the nature of the sugar units present.

**Acknowledgements**

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# III. ISOLAMENTO E IDENTIFICAÇÃO ESTRUTURAL DE COMPOSTOS TERPÉNICOS

# Índice

Publicação n.º 4 – "Terpenic compounds as chemical markers for *Cydonia oblonga* Miller"
(submetido para publicação) 93

# Terpenic compounds as chemical markers for *Cydonia oblonga* Miller

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In the course of a phytochemical study of *Cydonia oblonga* Miller, a terpenic compound (β-D-glucopyranoside of 9-amino-2,7-dimethyl-8-hydroxynona-2,4-dienoic acid) was isolated and identified by hydrolytic and spectroscopic means (nuclear magnetic resonance, mass spectroscopy and UV). Other chemically related compounds were also detected. Subsequently, these compounds were also quantified by HPLC/DAD in several pulps, peels and jams. These compounds are described for the first time in nature and can be used as a tool for the characterization of quince and its derivatives.

**Key words:** *Cydonia oblonga* Miller; quince; terpenic glycosides; chemical markers.

## INTRODUCTION

Quince fruits (*Cydonia oblonga Miller*), when ripe, are characterized by an agreeable, long lasting, and powerful flavor (*1*). As they are not edible due to their very hard, tough, and fibrous consistency, they are used for preparing jam. The powerful and characteristic odor of quince fruit is due to the presence of an essential oil (*1-2*) in which the characteristic compounds are thought to be monoterpenic lactones and oxides (*2-3*). In the early nineties Winterhalter and co-workers described the existence, in quince, of monoterpenic glucosides that were considered to be the precursors of the referred lactones and oxides (*4-5*).

Additionally, other phytochemical studies were made in quince (*6-15*). Several analytical methods were developed to determine phenolics, organic acids and free amino acids in quince fruit (pulp, peel, and seed) and jam, and their composition, in terms of these compounds, was established *(6-14)*. The influence of jam processing upon the contents of these compounds in quince fruit was also evaluated *(15)*.

In the course of our study of the phenolic profile of quince and its

derivatives, a few compounds noticed on the chromatograms recorded at 280 nm (with identical UV spectra when recorded with a DAD and maximum at approximately 270 nm) (*6-9*), remained unidentified. The paper herein discusses the chemical nature of the referred compounds. The amounts of three of the mentioned compounds, in several samples (quince pulps, peels and jams), were also determined by an HPLC/DAD method.

## MATERIALS AND METHODS

**Samples.** Healthy quince fruit samples were collected in different places in Northern (Amarante, Baião, Vila Real, Bragança, Custóias and Caminha) and Central Portugal (Viseu, Pinhel and Covilhã), in the years of 2000, 2001 and 2002. All fruits were separated in pulp and peel. Each part of the fruit was cut in thin slices and freeze-dried. Lyophilizations were carried out using a Labconco 4.5 apparatus (Kansas City, USA).

Quince jam samples from twenty brands were randomly purchased on the Portuguese market, in the years of 2000, 2001 and 2002, which included two types of manufacture - traditional (samples A - D) and industrial (samples E - T). A quince jam (jam U) was prepared in the laboratory by boiling fresh quince pulp (from Amarante, year of 2002) with sugar (in the proportion of 50:50), for approximately 90 min. Another quince jam (jam V) was similarly prepared, but using unpeeled quinces (from Amarante, year of 2002).

**Standards.** Citral was from Sigma (St. Louis, MO, USA) and methanol, formic and hydrochloric acids were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

**Isolation procedures.** For isolation purposes ca. 110 g of lyophilized quince pulp from Bragança (year of 2000) were used. Aliquots of 10 g of the powdered material were extracted twice with 750 ml of methanol, for 60 min, with agitation. The extracts were combined, filtered and the methanol was removed under reduced pressure (40 °C). The dried extract was thoroughly mixed with acidified water (pH 2 with HCl, 250 ml). Aliquots of 25 ml of this aqueous solution were passed through a column (300 x 30 mm) of Amberlite XAD-2 (Fluka Chemicals: pore size 9 nm, particle size 0.3 – 1.2 mm). The column was washed with acidified water (pH 2 with HCl, 200 ml) and subsequently with distilled water until neutral pH (ca. 1000 ml). Sugars and other polar compounds were eluted with the polar solvent. The compounds retained in the column were then eluted with

methanol, until complete extraction of phenolic compounds (ca. 500 ml), until negative reaction with NaOH 20%. The methanolic extract was concentrated to approximately 75 ml, at 40ºC, under reduced pressure and filtered. Aliquots of 15 ml of the concentrated extract were passed through a Sephadex LH-20 column (300 x 30 mm) (Sigma Chemical Co., St. Louis, MO: particle size 25 – 100 μm) with methanol, originating four fractions. The first fraction, with a light colour, and the fourth fraction, with a yellowish colour, were discarded after HPLC analysis (the first fraction contained mainly the already identified flavonoids and the fourth fraction contained mainly caffeoylquinic acids). Fractions 2 and 3, with a brownish color, were further separated in a LOBAR RP18 (310 x 25 mm; particle size 40 – 63 μm) column, using aliquots of 2.5 ml of the sample. The elution system was methanol / water starting with 10% methanol and with an increment of 10% every 10 min, until reaching 100% of methanol, which was kept for 60 min. Fractions were collected 40 min after the elution started, every 5 min, and analyzed by HPLC. The fractions collected between 1h 45 and 2h10 (collected each 10 min) were further purified by semi-preparative HPLC, using a reversed-phase Spherisorb ODS2 column (250 x 10 mm; 10 μm, particle size). The solvent system was water (A) and methanol (B) starting with 25% methanol, and installing a gradient to obtain 30% B at 18 min, 35% B at 30 min and 100% B at 34 min. Elution was performed at a solvent flow rate of 3.0 ml/min. The fractions were manually collected following UV detection at 280 nm. Three compounds were isolated: compound **a** eluted at 15.7 min, compound **b** at 18.9 min and compound **c** at 25.9 min.

**Acid treatment.** 1 ml of 2N HCl was added to 250 μl of each isolated compound solution or methanolic extract. The acid solution was heated at 100 ºC for 30 min and passed through an ISOLUTE C18 column (500 mg sorbent mass / 6 ml reservoir volume), previously conditioned with 5 ml of methanol and 5 ml of acid water (pH 2 with HCl) and the compounds were eluted with 5 ml of methanol. The methanolic extract was taken to dryness, redissolved in 250 ml of methanol, and 20 ml were analysed by HPLC.

**Alkaline treatment.** It was performed by adding 1 mL of 2N NaOH to 250 μl of each isolated compound solution or methanolic extract and keeping the mixture for 4 h at room temperature, in the dark. After this step, the alkaline hydrolysis products were acidified with concentrated HCl (up to pH

1-2) and passed through an ISOLUTE C18 column (500 mg sorbent mass / 6 ml reservoir volume), previously conditioned with 5 ml of methanol and 5 ml of acid water (pH 2 with HCl). The compounds were eluted with 5 ml of methanol. The methanolic extract was taken to dryness, redissolved in 250 ml of methanol, and 20 ml were analysed by HPLC.

**Spectrometric data of isolated compounds.** $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra were obtained on a Brucker AMX-300 instrument, operating at 500 and 125 MHz, respectively.

*β-D-glucopyranoside of 9-amino-8-hydroxi-2,7-dimethylnona-2,4-dienoic acid* (***a***). UV (MeOH): $\lambda_{máx}$ = 274 nm. $^{1}H$ NMR (DMSO-$d_6$): δ 0.80 (3H, d, $J$ = 6.6 Hz, $CH_3$-C7), 1.57 (1H, br s, H-C7), 1.87 (3H, s, $CH_3$-C2), 1.99 (1H, m, H-C6), 2.11 (1H, m, H-C9), 2.31 (1H, m, H-C9), 2.38 (1H, m, H-C6), 3.61 (2H, m), 5.39 (1H, d, $J$ = 7.9 Hz, H-C1'), 6.21 (1H, m, H-C5), 6.45 (1H, t, $J$ = 14.5, 11.7 Hz, H-C4), 7.21 (1H, d, $J$ = 11.3 Hz, H-C3). $^{13}C$ NMR (DMSO-$d_6$): δ 12.4 ($CH_3$ - 2), 15.4 ($CH_3$ – 7), 35.8 (C – 6), 38.6 (C – 7), 40.18 (C-9), 60.5 (C - 6'), 69.4 (C – 4'), 70.9 (C – 8), 72.5 (C – 2'), 76.4 (C – 3')[a], 77.8 (C – 5')[a], 94.5 (C – 1'), 123.6 (C – 2), 127.0 (C – 4), 139.8 (C – 3), 143.7 (C – 5), 166.4 (C – 1). [a]Assignments are interchangeable.

**Extraction for quantification purposes.** The extraction was achieved as previously reported (*8-9,14-15*) and included a C18 SPE cleaning step.

**HPLC analysis.** The extracts (20 µL) were analyzed as previously described (*6-9,14-15*), on an analytical HPLC unit (Gilson), using an Spherisorb ODS2 column (25.0 x 0.46 cm; 5µm, particle size). Detection was achieved with a Gilson DAD.

**Quantification**. In each extract, compounds **a** and **b** were localized by comparing their retention times and UV-Vis spectra, with those of the now isolated compounds. Peak purity was checked by means of the Gilson 160 spectra viewer software contrast facilities. Quantification of these compounds was achieved by the absorbance recorded in the chromatograms relative to external standard, with detection at 280 nm. All of the compounds were quantified as citral.

## RESULTS AND DISCUSSION

**Characterization of isolated compounds.** In previous studies concerning quince phenolics (*6-9*), some compounds, detected in the chromatograms recorded at 280 nm, remained unidentified (**Figure 1A**). They all showed a UV spectra with only one

maximum around 270 nm and, according to some published data, they were assigned as possible procyanidin polymers (*6*). A methanolic extract of quince pulp was now subjected to several procedures in order to isolate them and two compounds could be obtained in adequate purity to try the identification.

The UV spectra of compound **a** exhibited only one maximum at 274 nm. The results obtained from $^{1}H$ NMR spectrum indicated the presence of one methyl group in the aliphatic region (δ 0.80, 3H, d, $J$ = 6.6 Hz), another methyl group attached to an unsaturated carbon (δ 1.87, 3H, s), a conjugated alkene (δ 6.21, 1H, m; 6.45, 1H, t, $J$ = 14.5, 11.7 Hz; 7.21, 1H, d, $J$ = 11.3 Hz), four methylenic protons (δ 1.99, 1H, m; 2.08, 1H, m; 2.28, 1H, m; 2.37, 1H, m) and a methinic proton (δ 1.57, 1H, br s). Another methinic proton was detected at δ 5.39 ($J$ = 7.9 Hz) suggesting the presence of a β-glycosidic moiety in the molecule. In the $^{13}C$ NMR spectra, signals were detected for one carbonylic group (δ 166.4), two methyl groups (δ 12.4; 15.4), four carbon atoms involved in a conjugated alkene structure (δ 123.6; 127.0; 139.8; 143.7), one methinic carbon (δ 38.6) and two methylenic carbons (δ 35.8; 70.9). The high value found for carbon atom of the methylenic group at δ 70.9 indicated the existence of an oxygenated group. Other signals were found which corresponded to the carbons of a glycosidic structure (δ 60.5; 69.4; 72.5; 76.4; 77.8; 94.5), most probably glucose (*16*). The compound was also subjected to analysis by COSY, HSQC and HMBC in order to establish the correlations among the several portions of the molecule.

Analysis by HSQC showed that the methylenic protons at δ 2.08 and 2.28 were linked to a carbon whose chemical shift was overlapped by the signal of DMSO (δ around 40) but on HMBC no correlations were found between such carbon and any other protons of the molecule which raised some doubts about the exact chemical structure of the compound. On the other hand, several attempts to obtain a mass spectrum of compound **a** were unsuccessful.

In order to get a deep inside to the behavior of compound now isolated, it was subjected to acid treatment. Upon this treatment, compound **a** originated another compound with a higher retention time (**Figure 1B**) but the UV spectrum remained unchanged. This new compound was subjected to $^{1}H$ and $^{13}C$ NMR ($CDCl_3$). All the data obtained, including those from DEPT, COSY, HSQC and HMBC, showed that acid hydrolysis of compound **a** originated the compound represented in **Figure 2**. In the $^{13}C$ NMR

spectrum it was clearly found a carbon atom at δ 38.36 correlated (HSQC) with two methylenic protons (δ 2.40 and 2.50). On the other hand, carbon at δ 71.30 is correlated (HMBC) with the proton at 2.40, which means that the compound **a** now isolated from quince has, in fact, 11 carbon atoms. The presence of nitrogen on the molecule is deduced from the FAB/MS analysis of the aglycon, that exhibited a signal at m/z 214 indicating a mass of 213 u.m. Its presence is confirmed by the high chemical shift of the terminal carbon atom at δ 38.36. Glucose is linked to the aglycon through the carboxyl group given the behavior of the compound on alkaline hydrolysis and the downfield shift observed on the resonance of carbon 1 after hydrolysis. Attending to all these data compound **a** is identified as β-D-glucopyranoside of 9-amino-8-hydroxi-2,7-dimethylnona-2,4-dienoic acid.

The UV spectra of compound **c** exhibited only one maximum at 268 nm. Its $^{1}H$ NMR spectral properties were identical to those of compound **a**, except in the region δ 3.0 – 4.5. No signal around δ 5.0 was found indicating the lack of a glycosidic portion on the molecule. When subjected to the same acid treatment, compound **c** originated a peak, on HPLC, that was superimposable to the aglycon of compound **a**. The amount of isolated compound was insufficient for deeper studies, but given these data, compound **c** is most probably another ester of the compound represented on **Figure 2**.

Compound **b** was isolated in minute amounts and, when subjected to acid treatment, also originated a peak superimposable to the aglycon of compound **a.** All of these compounds originated the same peak after acid or alkaline hydrolysis.

Another peak on the chromatogram represented on **Figure 1A** showed UV spectra around 270 nm. **Figure 1B** shows the chromatogram of the methanolic extract after alkaline hydrolysis: on this chromatogram it can be observed the almost total disappearance of compounds **a**, **b** and **c** and that assigned with (·) and the appearance of a main compound at 39.9 min. This may be an indicative that other esters of the same acid are present on the methanolic extract of quince.

These compounds, although with a terpenic structure, are irregular terpenic compounds showing a "tail-to-tail" linking and an unusual number of carbons. This is the first report of a $C_{11}$ compound in quince, but $C_{13}$ and $C_{12}$ compounds are already described in this species (*17-18*). According to Lutz and

Winterhalter (*18*) such compounds can be considered as derived from the degradation of carotenoids: those with a $C_{13}$ ionol structure are derived from the terminal portions of the molecule, while those with a $C_{12}$ and $C_{10}$ structure, are derived from the central part of the carotenoids. The findings here reported reinforce such hypothesis.

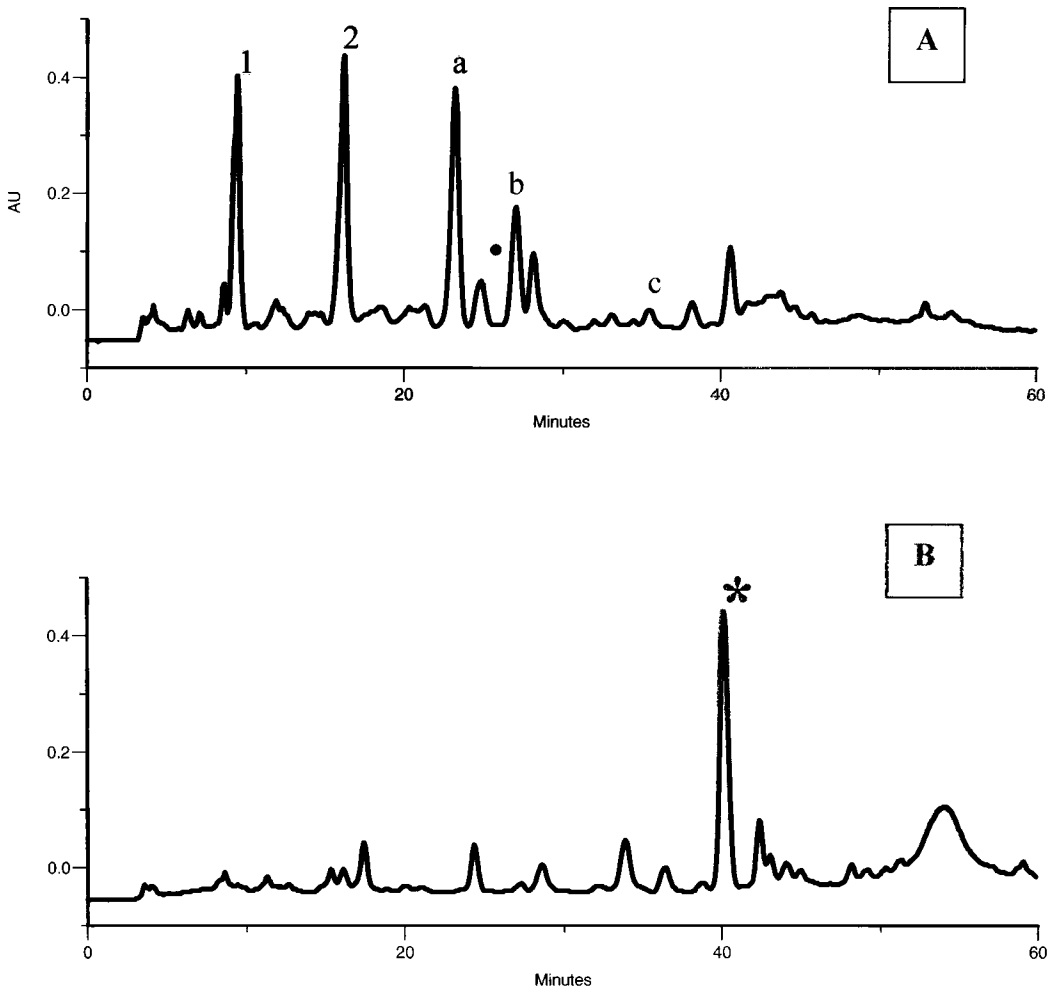


**Figure 1.** HPLC profile of the methanolic extract of quince pulp from Pinhel (2002) (A) and the results of the alkaline hydrolysis of the same extract (B). Detection at 280 nm. Peaks: (1) 3-*O*-caffeoylquinic acid, (2) 5-*O*-caffeoylquinic acid, (a), (b) and (c) isolated compounds, • compound with similar UV spectra and * resulting compound from alkaline hydrolysis.

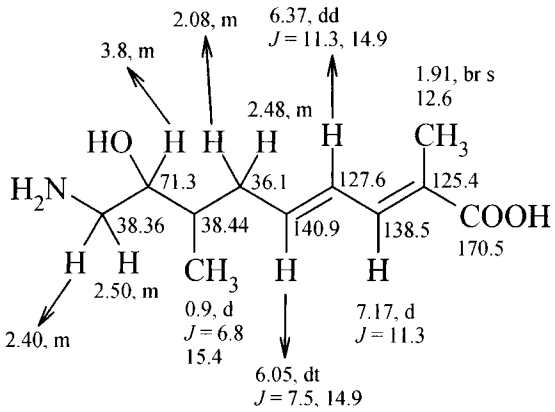


**Figure 2.** Chemical structure of the aglycon of compounds **a, b** and **c** isolated from *Cydonia oblonga* Miller.

The existence of a β-glucosidic ester of terpenic compound has also been reported in quince (*4*) but, contrarily to what happened to this ester, the compounds now described do not lactonize upon hydrolysis. Besides, this is the first report of a such compound including nitrogen in its molecule.

These compounds are easily observed on an HPLC chromatogram of methanolic extract of quince and can be considered as chemical makers, because they originate large peaks and that are not present in pear or apple (*6*).

**Quantitative analysis.** All analyzed samples (pulps, peels and jams) exhibited compounds **a**, **b** and **c** and their amounts are shown in **Tables 1-3**. Generally, compound **a** was present in higher amounts than the others. The mean contents of compounds **a** and **b** in pulps and peels were identical (59.0 and 59.5 mg/kg and 28.9 and 32.2 mg/kg, respectively). Compound **c** was present in higher amounts in peels than in pulps (24.9 and 14.2 mg/kg, respectively).

Jams generally had lower contents of these compounds. Nevertheless, jam U, that was prepared with pulp from Amarante (2002), showed higher amounts of compounds **a** than the correspondent pulp. This may also reinforce the hypothesis that these compounds are carotenoid-derived products. The cleavage of carotenoids may be favored by thermal processing, in the natural acid medium of quince.

**ACKNOWLEDGEMENT**

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**Table 1.** Isolated terpenes composition of quince pulps.

| | | Isolated compounds (mg/kg) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Geographic origin | Year | *Compound a*[a] | SD | *Compound b*[a] | SD | *Compound c*[a] | SD | Σ |
| Amarante | 2000 | 99.08 | 0.089 | 41.29 | 0.163 | 5.94 | 0.128 | 146.31 |
| | 2001 | 44.65 | 0.584 | 6.99 | 0.097 | 8.96 | 0.075 | 60.60 |
| | 2002 | 33.48 | 0.628 | 23.14 | 0.604 | 2.05 | 0.027 | 58.67 |
| Baião | 2000 | 65.40 | 0.432 | 31.43 | 0.675 | 5.05 | 0.151 | 101.88 |
| | 2001 | 59.96 | 0.857 | 15.17 | 0.037 | 10.21 | 0.009 | 85.34 |
| | 2002 | 51.54 | 1.999 | 38.70 | 0.515 | 20.08 | 0.117 | 110.32 |
| Vila Real | 2000 | 50.69 | 0.577 | 16.85 | 0.034 | 2.27 | 0.010 | 69.81 |
| | 2001 | 74.46 | 1.125 | 30.90 | 0.178 | 10.48 | 0.034 | 115.84 |
| | 2002 | 84.54 | 1.411 | 31.34 | 0.003 | 23.46 | 0.321 | 139.34 |
| Bragança | 2000 | 45.21 | 0.685 | 37.46 | 0.966 | 16.06 | 0.522 | 98.73 |
| | 2001 | 53.06 | 0.149 | 20.59 | 0.186 | 11.29 | 0.030 | 84.94 |
| | 2002 | 24.35 | 0.445 | 21.77 | 0.374 | 16.28 | 0.473 | 62.40 |
| Covilhã | 2000 | 48.32 | 0.222 | 24.54 | 1.453 | 5.36 | 0.088 | 78.22 |
| | 2001 | 100.78 | 0.446 | 36.26 | 1.962 | 14.05 | 0.054 | 151.09 |
| | 2002 | 37.30 | 1.957 | 16.81 | 0.479 | 36.69 | 0.809 | 90.80 |
| Viseu | 2000 | 59.82 | 1.062 | 29.53 | 0.384 | 5.89 | 0.048 | 95.24 |
| | 2001 | 90.79 | 2.202 | 49.41 | 1.413 | 21.90 | 0.116 | 162.10 |
| | 2002 | 59.24 | 0.560 | 28.38 | 0.529 | 28.61 | 0.321 | 116.23 |
| Pinhel | 2000 | 80.90 | 0.006 | 48.48 | 0.727 | 4.14 | 0.357 | 133.52 |
| | 2001 | 59.92 | 0.232 | 47.23 | 0.341 | 19.00 | 0.177 | 126.15 |
| | 2002 | 81.06 | 2.267 | 43.60 | 1.769 | 28.42 | 0.454 | 153.08 |
| Custóias | 2001 | 3.69 | 0.060 | 17.20 | 0.053 | 14.61 | 1.087 | 35.50 |
| | 2002 | 54.68 | 0.206 | 12.51 | 0.166 | 20.02 | 0.520 | 87.21 |
| Caminha | 2001 | 52.19 | 0.950 | 23.38 | 0.821 | 9.79 | 0.195 | 85.36 |
| mean | | 58.96 | | 28.87 | | 14.19 | | 102.03 |
| max | | 100.78 | | 49.41 | | 36.69 | | 162.10 |
| min | | 3.69 | | 6.99 | | 2.05 | | 35.50 |
| SD | | 23.20 | | 12.01 | | 9.17 | | 33.92 |

[a] values are expressed as mean of three determinations; SD – standard deviation; Σ - sum of the determined compounds.

**Table 2.** Isolated terpenes composition of quince peels.

| | | Isolated compounds (mg/kg) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Geographic origin | Year | *Compound a*[a] | SD | *Compound b*[a] | SD | *Compound c*[a] | SD | Σ |
| | 2000 | 105.87 | 4.169 | 52.37 | 2.756 | 23.53 | 0.714 | 181.77 |
| Amarante | 2001 | 56.24 | 0.145 | 22.82 | 0.445 | 17.40 | 0.140 | 96.46 |
| | 2002 | 32.40 | 0.650 | 23.94 | 0.647 | 9.04 | 0.430 | 65.38 |
| | 2000 | 51.11 | 0.221 | 43.12 | 0.178 | 15.77 | 0.027 | 110.00 |
| Baião | 2001 | 88.97 | 0.908 | 28.83 | 0.162 | 31.14 | 0.611 | 148.94 |
| | 2002 | 65.25 | 0.604 | 56.84 | 0.516 | 10.61 | 0.078 | 132.70 |
| | 2000 | 61.01 | 1.012 | 20.26 | 0.343 | 12.72 | 0.015 | 93.99 |
| Vila Real | 2001 | 61.18 | 0.357 | 22.34 | 0.035 | 33.33 | 1.116 | 116.85 |
| | 2002 | 56.00 | 0.896 | 25.16 | 0.233 | 16.90 | 1.333 | 98.06 |
| | 2000 | 35.31 | 1.789 | 42.90 | 1.417 | 8.71 | 0.141 | 86.92 |
| Bragança | 2001 | 36.38 | 0.396 | 9.04 | 0.637 | 25.89 | 1.141 | 71.31 |
| | 2002 | 26.11 | 0.487 | 24.35 | 0.489 | 17.07 | 0.745 | 67.53 |
| | 2000 | 37.12 | 0.806 | 37.95 | 0.473 | 20.07 | 0.082 | 95.14 |
| Covilhã | 2001 | 55.16 | 2.495 | 18.50 | 0.549 | 17.75 | 0.305 | 91.41 |
| | 2002 | 31.37 | 0.350 | 19.61 | 1.122 | 74.74 | 1.731 | 125.72 |
| | 2000 | 67.35 | 1.412 | 44.48 | 1.777 | 28.91 | 0.341 | 140.74 |
| Viseu | 2001 | 47.23 | 0.250 | 27.62 | 1.576 | 51.73 | 3.912 | 126.58 |
| | 2002 | 52.69 | 0.180 | 31.58 | 0.008 | 20.81 | 0.080 | 105.08 |
| | 2000 | 68.30 | 1.409 | 50.89 | 1.297 | 20.34 | 0.211 | 139.53 |
| Pinhel | 2001 | 52.41 | 2.405 | 25.93 | 1.982 | 30.65 | 0.733 | 108.99 |
| | 2002 | 44.27 | 0.009 | 28.32 | 0.217 | 14.12 | 0.781 | 86.71 |
| Custóias | 2001 | 111.38 | 1.750 | 55.02 | 0.538 | 51.05 | 0.080 | 217.45 |
| | 2002 | 55.00 | 1.532 | 16.65 | 0.539 | 6.14 | 0.261 | 77.79 |
| Caminha | 2001 | 129.83 | 0.498 | 45.85 | 0.150 | 38.86 | 0.289 | 214.54 |
| mean | | 59.50 | | 32.27 | | 24.89 | | 116.65 |
| max | | 129.83 | | 56.84 | | 74.74 | | 217.45 |
| min | | 26.11 | | 9.04 | | 6.14 | | 65.38 |
| SD | | 26.13 | | 13.52 | | 16.09 | | 41.47 |

[a] values are expressed as mean of three determinations; SD – standard deviation; Σ - sum of the determined compounds.

**Table 3.** Isolated terpenes composition of quince jams.

| | | Isolated compounds (mg/kg) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Jam | Year | *Compound a*[a] | SD | *Compound b*[a] | SD | *Compound c*[a] | SD | Σ |
| | 2000 | 17.16 | 0.252 | 12.58 | 0.182 | 26.49 | 1.082 | 56.23 |
| A | 2001 | 18.92 | 0.318 | 11.08 | 0.572 | 3.10 | 0.082 | 33.10 |
| | 2002 | 12.82 | 0.540 | 12.84 | 0.256 | 7.20 | 0.173 | 32.86 |
| B | 2000 | 25.78 | 0.179 | 24.61 | 0.814 | 17.00 | 0.806 | 67.39 |
| | 2001 | 30.64 | 0.044 | 11.33 | 0.253 | 4.66 | 0.002 | 46.63 |
| C | 2000 | 9.30 | 0.141 | 3.90 | 0.103 | 37.48 | 0.350 | 50.68 |
| | 2001 | 22.67 | 0.428 | 9.72 | 0.224 | 4.50 | 0.157 | 36.89 |
| D | 2000 | 38.93 | 0.628 | 18.92 | 0.418 | 17.18 | 0.199 | 75.03 |
| | 2000 | 29.49 | 1.242 | 18.52 | 0.142 | 5.43 | 0.178 | 53.44 |
| E | 2001 | 7.05 | 0.118 | 6.25 | 0.037 | 7.91 | 0.217 | 21.21 |
| | 2002 | 28.51 | 0.891 | 15.00 | 0.207 | 3.25 | 0.327 | 46.76 |
| | 2000 | 25.97 | 0.299 | 47.16 | 2.586 | 2.90 | 0.043 | 76.03 |
| F | 2001 | 18.55 | 0.273 | 13.99 | 0.035 | 2.74 | 0.019 | 35.28 |
| | 2002 | 15.99 | 0.360 | 12.60 | 0.122 | 2.77 | 0.048 | 31.36 |
| | 2000 | 22.42 | 0.698 | 83.26 | 0.337 | 3.86 | 0.095 | 109.54 |
| G | 2001 | 14.11 | 0.027 | 10.13 | 0.030 | 7.30 | 0.013 | 31.54 |
| | 2002 | 18.81 | 1.356 | 7.78 | 0.110 | 20.69 | 0.829 | 47.28 |
| H | 2000 | 20.26 | 1.629 | 24.99 | 1.667 | 2.66 | 0.079 | 47.91 |
| | 2001 | 6.04 | 0.097 | 5.75 | 0.094 | 33.59 | 0.183 | 45.38 |
| | 2000 | 20.72 | 0.596 | 12.93 | 0.375 | 3.10 | 0.134 | 36.75 |
| I | 2001 | 17.76 | 0.426 | 14.61 | 0.084 | 17.50 | 0.413 | 49.87 |
| | 2002 | 30.28 | 2.652 | 21.58 | 0.610 | 3.27 | 0.337 | 55.13 |
| | 2000 | 32.44 | 0.396 | 22.10 | 0.460 | 3.16 | 0.107 | 57.70 |
| J | 2001 | 13.13 | 0.021 | 18.11 | 0.513 | 5.21 | 0.182 | 36.45 |
| | 2002 | 8.55 | 0.141 | 8.88 | 0.114 | 8.84 | 0.117 | 26.27 |
| | 2000 | 33.73 | 0.275 | 31.98 | 0.184 | 3.85 | 0.234 | 69.56 |
| K | 2001 | 24.81 | 0.117 | 14.34 | 0.010 | 2.44 | 0.223 | 41.59 |
| | 2002 | 31.36 | 2.428 | 20.52 | 0.666 | 1.92 | 0.019 | 53.80 |
| L | 2000 | 18.00 | 1.370 | 22.60 | 0.035 | 1.77 | 0.001 | 42.37 |
| | 2000 | 42.38 | 1.555 | 23.58 | 0.269 | 5.49 | 0.045 | 71.45 |
| M | 2001 | 11.05 | 0.158 | 14.75 | 0.456 | 25.39 | 0.349 | 51.19 |
| | 2002 | 12.93 | 0.029 | 11.22 | 0.130 | 18.59 | 0.924 | 42.74 |
| | 2000 | 11.37 | 0.801 | 41.96 | 2.469 | 11.05 | 0.520 | 64.38 |
| N | 2001 | 19.27 | 0.020 | 19.66 | 0.159 | 21.01 | 0.687 | 59.94 |
| | 2002 | 34.37 | 1.231 | 25.21 | 1.764 | 5.38 | 0.120 | 64.96 |
| | 2000 | 10.84 | 0.563 | 28.33 | 0.527 | 12.08 | 0.159 | 51.25 |
| O | 2001 | 16.42 | 0.561 | 12.00 | 0.399 | 1.50 | 0.076 | 29.92 |
| | 2002 | 25.58 | 0.004 | 20.36 | 0.065 | 15.65 | 0.342 | 61.59 |
| | 2000 | 14.31 | 0.130 | 28.69 | 0.122 | 1.23 | 0.040 | 44.23 |
| P | 2001 | 10.24 | 0.064 | 8.86 | 0.240 | 2.93 | 0.025 | 22.03 |
| | 2002 | 15.34 | 0.069 | 12.28 | 0.075 | 11.36 | 0.100 | 38.98 |
| | 2000 | 28.08 | 0.113 | 36.66 | 0.373 | 3.53 | 0.047 | 68.27 |
| Q | 2001 | 24.82 | 0.285 | 15.17 | 0.063 | 9.83 | 0.031 | 49.82 |
| | 2002 | 33.46 | 0.526 | 20.40 | 0.081 | 5.06 | 0.086 | 58.92 |
| | 2000 | 39.92 | 0.234 | 69.11 | 0.158 | 34.90 | 2.362 | 143.93 |
| R | 2001 | 10.93 | 0.045 | 8.19 | 0.334 | 7.92 | 0.220 | 27.04 |
| | 2002 | 23.93 | 0.970 | 15.76 | 0.257 | 2.50 | 0.002 | 42.19 |
| S | 2000 | 31.94 | 0.319 | 42.53 | 0.425 | 4.54 | 0.054 | 79.01 |
| T | 2000 | 20.19 | 0.071 | 73.15 | 2.299 | 4.99 | 0.102 | 98.33 |
| U | 2002 | 46.01 | 0.204 | 18.02 | 0.199 | 2.54 | 0.172 | 66.57 |
| V | 2002 | 24.92 | 0.249 | 16.42 | 0.185 | 2.14 | 0.185 | 43.48 |
| mean | | 22.01 | | 21.58 | | 9.24 | | 52.83 |
| max | | 46.01 | | 83.26 | | 37.48 | | 143.93 |
| min | | 6.04 | | 3.90 | | 1.23 | | 21.21 |
| SD | | 9.68 | | 16.54 | | 9.26 | | 22.23 |

[a] values are expressed as mean of three determinations; SD – standard deviation; Σ - sum of the determined compounds.

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# IV. PERFIL EM ÁCIDOS ORGÂNICOS

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# Study of the Organic Acids Composition of Quince (*Cydonia oblonga* Miller) Fruit and Jam

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The organic acids present in several samples of quince fruit (pulp and peel) and quince jam (homemade and industrially manufactured) were analyzed by HPLC. The sample preparation was simple, involving only extraction with methanol (40 °C) and filtration through a Sep-pack C18 cartridge. The chromatographic separation was achieved using an ion exclusion column, Nucleogel Ion 300 OA (300 × 7.7 mm), in conjunction with a column heating device at 30 °C. An isocratic elution with $H_2SO_4$ 0.01 N as the mobile phase, with a flow rate of 0.1 mL/min, and UV detection at 214 nm were used. These analyses showed that all samples presented a similar profile composed of at least six identified organic acids: citric, ascorbic, malic, quinic, shikimic, and fumaric acids. Several samples also contained oxalic acid. This study suggests that the organic acids levels and ratios may be useful for the determination of percent fruit content of quince jams. The citric acid value can also be used in the differentiation of the type of manufacture of the commercial quince jams (homemade or industrially manufactured).

**KEYWORDS:** ***Cydonia oblonga* Miller; quince fruit; quince jam; HPLC−UV; organic acids**

## INTRODUCTION

Quince is the fruit of a deciduous tree of the *Rosaceae* family, *Cydonia oblonga* Miller. When ripe, quince fruits impart an agreeable, long-lasting, and powerful flavor. As they are not edible unprocessed because of their very hard, tough, and fibrous consistency, they are often used for preparing jam (*1*). Quince jam, designated "marmelada", is a compote with great tradition in Portugal. Because quince is a seasonal fruit, its homemade jam is prepared during September/October by boiling a mixture of sugar and quince fruits, normally in the proportion of 50:50, until the appropriate consistency is reached (usually 65−72 °Brix) (*2*). The industrially manufactured quince jam is prepared with quince puree, sugar, and additives (preservatives such as benzoic and sorbic acids; antioxidants such as ascorbic acid; and acidity regulators such as citric and tartaric acids; etc.).

The nature and the concentration of organic acids are important factors influencing the organoleptic properties of fruit and fruit products (*3*). Accurate knowledge of organic acid levels (and ratios) is sometimes useful to determine the percent fruit content of fruit products, and also to detect adulteration in this food class (*4*). Acids are components of fruits that have a lower susceptibility to change during processing and storage than other components such as pigments and flavor compounds. This relative stability offers a practical advantage for using an organic acids profile as an index of authenticity in fruit products (*5*, *6*). However, separation and accurate quantification of individual organic acids in these products have been considered difficult because of their structural similarities and lack of distinctive spectral properties (*3*). Furthermore, most of the organic acids of interest in fruit products are weak acids with similar $pK_a$ values. Because of these similarities, their chromatographic behaviors are identical, and therefore they cannot be separated quantitatively if one of the acids is present in relatively large amounts (*3*).

For quince fruit and its derivatives, few studies have been developed. As far as we know, there is no study about organic acids composition of quince fruit and/or its jam. So, the work herein represents a contribution for the definition of the organic acids profile of the pulp and peel from this fruit and its jam. With this purpose, samples of quince fruit from seven different geographic origins from Portugal, as well as twenty commercial quince jam samples (four homemade and sixteen industrially manufactured) were analyzed. A quince jam was also prepared with pulps of fruits from one of the geographic origins analyzed to check the usefulness of organic acids composition in the determination of the percent fruit content of quince jams.

## MATERIALS AND METHODS

**Samples.** Healthy quince fruit samples were collected in different places in Northern (Amarante, Baião, Vila Real, and Bragança) and Central Portugal (Viseu, Pinhel, and Covilhã). All fruits were separated into pulp and peel. Each part of the fruit was cut into thin slices and freeze-dried.

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A quince jam was prepared in the laboratory by boiling quince pulps from Amarante with sugar (in the proportion of 50:50) during approximately 60 min.

Twenty commercial quince jam samples, randomly purchased on the Portuguese market, were assayed, and these included four that were homemade (samples A−D) and sixteen that were industrially manufactured (samples E−T).

**Standards.** The standards were from Sigma (St. Louis, MO) and from Extrasynthése (Genay, France). Methanol and hydrochloric acid were obtained from Merck (Darmstadt, Germany), and sulfuric acid was obtained from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

**Solid-Phase Extraction (SPE) Columns.** The ISOLUTE C18 non end-capped (NEC) SPE columns (50 $\mu$m particle size, 60 Å porosity; 10 g sorbent mass/70 mL reservoir volume) were purchased from International Sorbent Technology Ltd (Mid Glamorgan, UK).

**Extraction of Organic Acids.** Each sample (ca. 3 g for quince jams and 1 g for quince pulps and peels) was thoroughly mixed with methanol (10 × 50 mL) or acid water (pH = 2 with HCl). Two different temperatures of extraction were assayed: ambient and 40 °C. The methanolic extract was filtered, concentrated to dryness under reduced pressure (40 °C), and redissolved in acid water (pH 2 with HCl) (ca. 50 mL). The aqueous solutions obtained were then passed through an ISOLUTE C18 (NEC) column, previously conditioned with 30 mL of methanol and 70 mL of acid water (pH 2 with HCl). The aqueous extracts were evaporated to dryness under reduced pressure (40 °C) and redissolved in sulfuric acid 0.01 N (3 mL), and 20-$\mu$L samples were analyzed by HPLC.

**HPLC Analysis of Organic Acids.** The separation of organic acids was achieved with an analytical HPLC unit (Gilson), using an ion exclusion column (Nucleogel Ion 300 OA; 300 × 7.7 mm) or a Spherisorb ODS2 column (25.0 × 0.46 cm; 5$\mu$m, particle size) in conjunction with a column heating device at 25, 30, 40, 50, 60, and 70 °C. Elution was carried out isocratically with sulfuric acid (0.01, 0.005, and 0.0025 N) as the mobile phase. The following flow rates were assayed: 0.1, 0.2, 0.3, 0.4, and 0.5 mL/min. 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. Malic and quinic acids were quantified together and as malic acid.

**Statistics.** Data are presented as the mean ± standard deviation. For statistical evaluation of citric and ascorbic acids contents from industrially manufactured and homemade quince jams a one-way analysis of variance (ANOVA) was used. Means evaluated were considered significantly different at $p < 0.05$.

## RESULTS AND DISCUSSION

To optimize the HPLC conditions for analysis of organic acids from quince fruit and its jam, an artificial mixture was prepared containing thirteen acids previously reported from other fruits (especially those from pear and apple which also belong to the *Rosaceae* family) (*3*−*14*): oxalic, *cis*-aconitic, citric, ascorbic, α-ketoglutaric, pyruvic, malic, quinic, succinic, lactic, shikimic, fumaric, and tartaric, as there are no studies on the organic acids composition of quince. To choose the best conditions of analysis, several methodologies of separation were tried, and the best results were obtained using an ion exclusion Nucleogel Ion 300 OA (300 × 7.7 mm) column, in conjunction with a column heating device at 30 °C, and isocratic elution with sulfuric acid 0.01 N, at a 0.1 mL/min flow rate. This chromatographic procedure has the advantage of allowing the detection of many of the organic acids of interest in quince fruit and its jam without precolumn derivatization.

From the assayed extraction conditions, higher yields were obtained using methanol at 40 °C. The filtration through a Sep-pack C18 (NEC) cartridge eliminates interference compounds, such as phenolics, that are retained by the sorbent.

All samples presented a similar profile composed of, at least, six identified organic acids: citric, ascorbic, malic, quinic, shikimic and fumaric acids. Several of them also contained oxalic acid. None of the samples revealed the presence of *cis*-aconitic, α-ketoglutaric, pyruvic, succinic, lactic, or tartaric acids.

Under the assay conditions described, a linear relationship between the concentration of oxalic, citric, ascorbic, malic, shikimic, and fumaric acids and the UV absorbance at 214 nm was obtained. The correlation coefficient for the standard curves invariably exceeded 0.99 for all organic acids. The calibration curves were obtained by triplicate determinations of each of the calibration standards, and the peak area values (arbitrary units) were plotted as average values. The relative percent average deviations of triplicates were less than 2% in all cases. The average regression equation for oxalic, citric, ascorbic, malic, shikimic, and fumaric acids were $y = 5.27 \times 10^5x$, $y = 7.90 \times 10^7x$, $y = 1.33 \times 10^7x$, $y = 5.95 \times 10^7x$, $y = 4.84 \times 10^6x$, and $y = 1.05 \times 10^{10}x$, respectively.

The detection limit values were calculated as the concentration corresponding to three times the standard deviation of the background noise, and the values obtained ranged from 0.01 to 1.67 $\mu$g/mL. The precision of the analytical method was evaluated by measuring the peak chromatographic area of organic acids six times on the same sample. The analytical method is precise: the coefficients of variation of organic acids were between 1.52 and 5.36%.

To study the recovery of the procedure, one quince pulp, one peel, and one jam sample were added to known quantities of oxalic, citric, ascorbic, malic, shikimic, and fumaric acids. The samples were analyzed in triplicate before and after the additions. Recovery values were high, between 82.9 and 112.1%, which demonstrates the effectiveness of the extraction and the accuracy of the method.

**Quince Fruits.** As previously stated, all quince pulps and peels presented citric, ascorbic, malic, quinic, shikimic, and fumaric acids (**Table 1**). Several samples also contained oxalic acid. The sum of all quantified acids ranged from ca. 7 to 14 g/kg, either in pulps or in peels. In some cases (Amarante, Vila Real, Bragança, and Pinhel) the amount of acids was higher in pulps than in the corresponding peels; in other cases the differences were very small, and even in one case (Baião) the peels were richer than the pulps. As can be seen in **Table 1**, the total amounts of organic acids were related to the quince fruit geographical origin. However, other factors, such as cultural practices and degree of maturation, may have caused the observed differences.

Despite this variability, there are features common to all analyzed samples. In all cases the sum of malic acid plus quinic acid represented 96 to 99% of the total acid content in pulps and 93 to 98% in peels. That fact means that all other acids were present in very small amounts, usually lower than 1%, with the exception of citric acid which ranged from traces to 6%.

**Quince Jams.** To test the usefulness of the knowledge of organic acids levels and their ratios in the determination of the percent fruit content, and to study the behavior of the acids during processing, a quince jam was prepared in the laboratory by boiling pulps of fruits from Amarante and sugar (50:50). On comparing the total acids amount of this jam with that of the used pulp, it can be seen that it corresponds to approximately 52%, which is very similar to the percent fruit content utilized (50%). Moreover, the ratios among the acids stayed roughly

**Table 1.** Organic Acids Composition of Pulp and Peel from Quince Fruit[a]

| | organic acids (mg/kg) | | | | | | |
|---|---|---|---|---|---|---|---|
| geographic origin | oxalic acid (RT 39.56 min) | citric acid (RT 53.83 min) | ascorbic acid (RT 57.50 min) | malic + quinic acids (RTs 64.93, 67.57 min) | shikimic acid (RT 85.48 min) | fumaric acid (RT 117.34 min) | Σ |
| | | | | pulp | | | |
| Amarante | 9.1 (0.16) | 179.9 (1.69) | 31.2 (1.32) | 10302.2 (132.96) | 14.2 (0.16) | tr | 10536.6 |
| Baião | 3.0 (0.08) | tr | 158.1 (5.43) | 6725.2 (135.81) | 15.0 (0.13) | 0.1 (0.01) | 6901.4 |
| Vila Real | tr | 240.8 (2.03) | 38.5 (0.94) | 10985.7 (64.66) | 19.3 (0.06) | 0.1 (0.01) | 11284.4 |
| Bragança | nd | 14.7 (0.41) | 116.8 (5.38) | 12642.9 (133.46) | 11.1 (0.01) | 0.9 (0.01) | 12786.4 |
| Covilhã | 5.0 (0.14) | 558.8 (12.69) | 15.8 (2.08) | 13358.3 (310.63) | 24.1 (0.29) | 0.1 (0.01) | 13962.1 |
| Viseu | nd | 253.4 (2.33) | 37.9 (0.79) | 9380.2 (64.46) | 18.5 (0.01) | 0.1 (0.01) | 9690.1 |
| Pinhel | 6.8 (0.40) | 55.6 (0.92) | 45.7 (1.84) | 14057.7 (100.92) | 19.8 (0.03) | 0.2 (0.01) | 14185.8 |
| min | nd | **tr** | **15.8** | **6725.2** | **11.1** | **tr** | 6901.4 |
| max | 9.1 | **558.8** | **158.1** | 14057.7 | **24.1** | **0.9** | 14185.8 |
| mean | **3.4** | 186.2 | 63.4 | 11064.6 | **17.4** | **0.2** | 11335.3 |
| SD | **3.68** | 194.72 | 52.77 | 2548.39 | **4.30** | **0.31** | 2586.43 |
| | | | | peel | | | |
| Amarante | 10.9 (0.23) | 66.0 (1.83) | 122.2 (11.57) | 8771.6 (106.21) | 17.1 (0.10) | tr | 8987.8 |
| Baião | nd | 269.5 (3.97) | 43.8 (3.49) | 13174.1 (266.97) | 23.1 (0.36) | 0.6 (0.02) | 13511.1 |
| Vila Real | 12.1 (0.55) | 92.5 (6.08) | 150.2 (2.03) | 8873.7 (346.21) | 26.0 (1.03) | 5.9 (0.02) | 9160.4 |
| Bragança | tr | 47.9 (3.23) | 124.4 (2.09) | 7565.9 (338.33) | 18.6 (0.15) | 1.1 (0.01) | 7757.9 |
| Covilhã | tr | 140.2 (0.92) | 73.8 (3.67) | 13727.8 (1.29) | 32.7 (0.75) | 0.1 (0.01) | 13974.6 |
| Viseu | nd | 204.0 (5.60) | 126.7 (1.75) | 10410.9 (175.53) | 27.2 (0.01) | 0.5 (0.02) | 10769.3 |
| Pinhel | nd | 792.6 (99.67) | 76.5 (1.57) | 12606.6 (438.36) | 20.7 (0.32) | 0.4 (0.05) | 13496.8 |
| min | nd | **47.9** | **43.8** | 7565.9 | **17.1** | **tr** | 7757.9 |
| max | 12.1 | **792.6** | **150.2** | 13727.8 | **32.7** | **5.9** | 13974.6 |
| mean | 3.3 | **230.4** | **102.5** | 10732.9 | **23.6** | **1.2** | 11094.0 |
| SD | 5.62 | **260.06** | **38.02** | 2445.29 | **5.44** | **2.09** | 2560.06 |

[a] Values are expressed as mean (standard deviation) of three determinations; tr, traces; nd, not detected; Σ, sum of the determined organic acids; RT, retention time.

**Table 2.** Organic Acids Composition of Quince Pulp from Amarante and Its Jam[a]

| | organic acids (mg/kg) | | | | | | |
|---|---|---|---|---|---|---|---|
| samples | oxalic acid (RT 39.56 min) | citric acid (RT 53.83 min) | ascorbic acid (RT 57.50 min) | malic + quinic acids (RTs 64.93, 67.57 min) | shikimic acid (RT 85.48 min) | fumaric acid (RT 117.34 min) | Σ |
| pulp | 9.1 (0.16) | 179.9 (1.69) | 31.2 (1.32) | 10302.2 (132.96) | 14.2 (0.16) | tr | 10536.6 |
| jam | 7.0 (0.41) | 91.4 (0.22) | 14.8 (0.88) | 5414.7 (111.86) | 5.3 (0.06) | tr | 5533.2 |

[a] Values are expressed as mean (standard deviation) of three determinations; tr, traces; Σ, sum of the determined organic acids; RT, retention time.

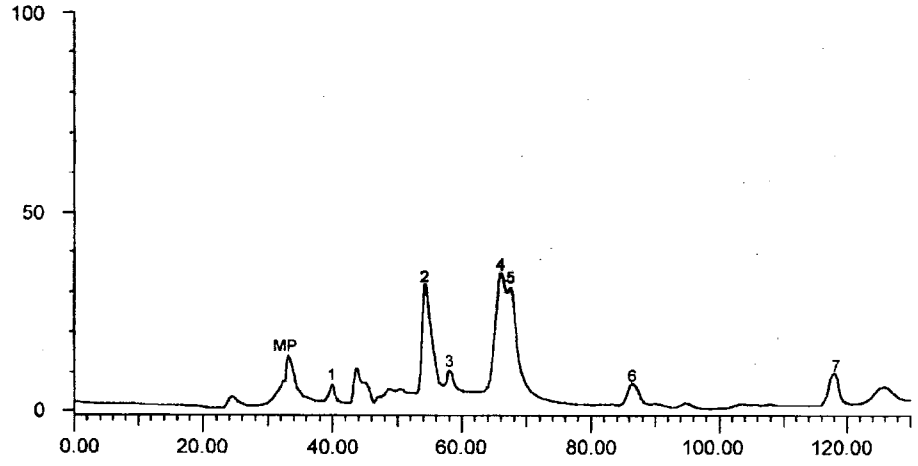


**Figure 1.** HPLC organic acids profile of a quince jam, detection at 214 nm: (MP) mobile phase; (1) oxalic acid; (2) citric acid; (3) ascorbic acid; (4) malic acid; (5) quinic acid; (6) shikimic acid; (7) fumaric acid.

constant, even for ascorbic acid, the most thermolabile of the detected acids (**Table 2**).

Twenty commercial quince jam samples were analyzed by the proposed technique, and **Figure 1** shows a typical HPLC profile of an industrially manufactured quince jam sample. In a general way, as it happened with quince fruits, either in the homemade quince jams (samples A−D) or in the industrially manufactured ones (samples E−T), the major peaks in the chromatograms correspond to the sum of malic and quinic acids (**Table 3**).

**Table 3.** Organic Acids Composition of Commercial Quince Jam Samples[a]

| | organic acids (mg/kg) | | | | | | |
|---|---|---|---|---|---|---|---|
| sample | oxalic acid (RT 39.56 min) | citric acid (RT 53.83 min) | ascorbic acid (RT 57.50 min) | malic + quinic acids (RTs 64.93, 67.57 min) | shikimic acid (RT 85.48 min) | fumaric acid (RT 117.34 min) | Σ |
| A[b] | tr | 4.6 (0.11) | 101.0 (4.36) | 5303.3 (81.88) | 7.5 (0.16) | 1.6 (0.20) | 5418.0 |
| B[b] | nd | 3.7 (0.13) | 158.8 (26.10) | 5697.8 (498.59) | 6.2 (0.21) | 1.3 (0.07) | 5867.8 |
| C[b] | nd | tr | 426.0 (48.52) | 2280.9 (69.55) | 5.7 (0.45) | 0.1 (0.01) | 2712.6 |
| D[b] | tr | tr | 120.6 (3.64) | 2385.4 (63.84) | 8.1 (0.62) | tr | 2514.1 |
| E[c] | 37.3 (2.00) | 1758.1 (26.67) | 208.1 (5.35) | 976.2 (51.97) | 7.8 (0.36) | 0.1 (0.01) | 2987.6 |
| F[c] | 1.4 (0.02) | 876.2 (6.79) | 207.9 (2.79) | 3921.5 (38.48) | 5.5 (0.01) | 0.1 (0.02) | 5012.5 |
| G[c] | 3.0 (0.10) | 1425.6 (22.45) | 68.3 (10.29) | 2931.1 (96.67) | 0.9 (0.06) | 0.1 (0.02) | 4428.9 |
| H[c] | tr | 710.1 (8.39) | 103.0 (9.45) | 4684.1 (59.43) | 14.8 (0.01) | tr | 5512.0 |
| I[c] | 2.3 (0.01) | 944.3 (7.18) | 153.1 (3.86) | 5778.5 (236.08) | 7.8 (0.13) | 0.1 (0.01) | 6886.0 |
| J[c] | 3.5 (0.05) | 1184.5 (35.87) | 793.7 (26.26) | 4124.2 (169.83) | 10.8 (0.03) | 1.2 (0.21) | 6117.9 |
| K[c] | tr | 825.5 (45.99) | 115.8 (9.68) | 6539.3 (558.15) | 8.9 (0.01) | 0.6 (0.04) | 7490.1 |
| L[c] | tr | 817.7 (4.46) | 84.3 (4.13) | 2194.2 (240.64) | 13.2 (0.13) | 0.1 (0.01) | 3109.4 |
| M[c] | nd | 1150.1 (64.08) | 176.3 (1.54) | 8077.6 (25.33) | 13.9 (0.05) | 2.1 (0.01) | 9420.0 |
| N[c] | 1.8 (0.12) | 229.8 (9.09) | 130.3 (7.86) | 5464.2 (91.19) | 9.1 (0.01) | 1.2 (0.05) | 5836.4 |
| O[c] | 2.6 (0.08) | 1089.4 (15.82) | 165.2 (24.43) | 10059.7 (27.74) | 14.3 (0.02) | 0.2 (0.01) | 11331.4 |
| P[c] | 12.1 (0.07) | 3096.6 (17.33) | tr | 9061.5 (564.30) | 7.2 (0.16) | tr | 12177.5 |
| Q[c] | nd | 210.4 (2.07) | 144.0 (17.34) | 11684.2 (284.11) | 13.2 (0.24) | tr | 12051.8 |
| R[c] | 5.7 (0.12) | 1849.8 (82.50) | tr | 2045.0 (96.59) | 6.9 (0.05) | 0.1 (0.01) | 3907.4 |
| S[c] | tr | 993.5 (68.38) | 225.9 (20.18) | 3185.9 (117.52) | 30.5 (0.16) | 0.1 (0.01) | 4435.8 |
| T[c] | 1.5 (0.00) | 3860.9 (65.28) | 107.3 (10.60) | 3812.7 (37.98) | 9.2 (0.09) | 1.7 (0.27) | 7793.2 |
| min | nd | tr | tr | 976.2 | 0.9 | tr | 2514.1 |
| max | 37.3 | 3860.9 | 793.7 | 11684.2 | 30.5 | 2.1 | 12177.5 |
| mean | 3.6 | 1051.5 | 174.5 | 5010.4 | 10.1 | 0.5 | 6250.5 |
| SD | 8.45 | 1011.41 | 171.21 | 2884.17 | 5.95 | 0.70 | 3001.44 |

[a] Values are expressed as mean (standard deviation) of three determinations. tr, traces; nd, not detected; SD, standard deviation; Σ, sum of the determined organic acids; RT, retention time. [b] Homemade quince jam samples. [c] Industrially manufactured quince jam samples.

The homemade samples maintained a profile very similar to that observed for quince fruits, except for jam C, where ascorbic acid reached ca. 16%. This could be due to an addition of ascorbic acid to this quince jam, although the fact was not mentioned on the label.

In the industrially manufactured jams the total acid content ranged from 3 to 12 g/kg, but the ratio among the acids was altered. This is explained by the addition of citric and ascorbic acids (as mentioned on the label, although the amounts were not provided) as acidity regulator and antioxidant, respectively.

Regarding the citric acid contents, there are significant differences between the two manufacture types ($F = 7.12$, $p = 0.05$): in the homemade quince jams this acid is presented in very low content (mean value of 2.1 mg/kg; $n = 4$), when compared to industrially manufactured jams (mean value of 1313.9 mg/kg; $n = 16$). So, it is possible to distinguish the quince jams type of manufacture (industrial or homemade) by this parameter.

The difference between the mean value of ascorbic acid content from homemade (201.6 mg/kg; $n = 4$) and industrially manufactured quince jams (167.7 mg/kg; $n = 16$) was not significant ($p > 0.05$).

All the organic acids herein detected in quince were already described in apple and pear: two fruits also belonging to the same botanical family (*Rosaceae*) and frequently used to adulterate quince jams. So, contrarily to what happens with the phenolic profile (*2*), it is not possible to detect any of these falsifications by the qualitative analysis of organic acids. The detection of such adulteration by the analysis of the acids ratios does not seem very feasible: first, because, as far as we know, there is no accurate knowledge about the acids ratios in these fruits; and second, because, as already mentioned, the law allows the addition of citric and ascorbic acids. Despite this, the study of the organic acids ratios allows an approximate evaluation of the amounts of the added acids. When analyzing **Table 3** under this point of view, we are induced to suspect that some of the jams, namely sample E, were adulterated. This sample has a very low content of malic plus quinic acids and a high amount of citric acid. Maybe this high addition of citric acid was intended to mask a low amount of quince, while maintaining the pH within the legal values (between 2.8 and 3.5) (*15*). So, knowledge of the composition of quince fruit and jam in terms of organic acids may help in the quality control of those products.

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# Development and Evaluation of a GC/FID Method for the Analysis of Free Amino Acids in Quince Fruit and Jam

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A GC/FID methodology for determination of twenty-one free amino acids in quince fruit (pulp and peel) and jam is described. The sample preparation was simple, involving a SCX Solid-Phase Extraction (SPE) purification step and a fast derivatization with ethyl chloroformate for gas chromatographic analysis. The chromatographic separation was achieved using a CP-Sil 19 CB wcot fused-silica capillary column. Under the chosen conditions, with temperature and pressure programming, this capillary column was able to separate all the amino acids not only in a short time but also with good separation. The GC/FID procedure is rapid, sensitive, reproducible and accurate. The detection limit values for amino acids were low, between 0.004 and 0.115 μg/mL, and the method was precise. As a general rule, the recovery values were high. Due to its rapidity and low cost, this technique can be useful in the quality control of quince products.

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Amino acids are present in almost all foods, so a foodstuff may often be characterized by its relative amounts (fruit juices, syrups, jams and jellies; meat and meat products; wines).[1] In plants and plant materials, in fruit and their products such as juices, jams and jellies, they are found in the free form, mainly in the L-configuration.

The applications of the amino acids analysis are nearly unlimited and may roughly be divided into three main groups:[1] determination of the chemical score of food proteins; influence of technological processes and additives on that score; detection of fraudulent manipulations or economically profitable falsifications, especially in fruit products.

Generally, the analysis of amino acids in food matrices is done by liquid chromatography.[2] Gas chromatography (GC) has, however, some advantages, given its capillary flexibility, higher resolution, speed of analysis and even its instrumental cost that is about one third of that of HPLC. Nevertheless, GC analysis of amino acids require a derivatization step, which commonly involves laborious and multi-step procedures, and this limitation greatly diminishes the above-cited advantages of GC.[3] This problem was solved by Husek,[3-6] who developed rapid derivatization procedures based on the treatment of amino acids with chloroformates.

Quince is the fruit of a deciduous tree of the *Rosaceae* family, *Cydonia oblonga* Miller. This fruit is too acid, astringent, and tough to be consumed fresh. However, it can be consumed when cooked or processed as jam or jelly, two food products with long tradition in Portugal. Quince jam is homemade or industrially manufactured, by boiling a mixture of sugar and quince puree until a convenient texture is obtained (usually to reach 65–72°Brix). When quince production is scarce, industry manufacturers are tempted to adulterate quince jam by adding apples and/or pears due to their low cost.

For quince fruit and its derivatives, few studies have been developed. In 1979 and 1986, the volatile constituents of quince fruit were analyzed by GC/MS.[7,8] Later, the usefulness of phenolic compounds in the determination of genuineness of quince puree,[9] jam[10,11] and jelly[12] has been reported. Glucosides of procyanidin polymers have been previously identified in this fruit[13,14] and, recently, it was possible to discriminate quince pulp and peel by the analysis of phenolic compounds in quince fruit.[15] In 2002, an HPLC/UV method was developed for the determination of organic acids in quince fruit and its jam.[16] As far as we know, there is no study about amino acids composition of quince fruit and jam. So, once all fruits show a typical free amino acids pattern,[1] the aim of the present study was to develop an GC analytical technique for the qualitative and quantitative analysis of free amino acids in quince fruit (pulp and peel) and jam.

## Experimental

*Samples and standards*

The L-amino acids and the internal standard (L-*p*-chlorophenylalanine) were all from Sigma (St. Louis, MO, USA). Ethyl chloroformate (ECF) was from Aldrich (Steinheim, Germany) and pyridine from Fluka (Neu-Ulm, Germany). All other chemicals were of analytical grade from several suppliers.

Healthy quince fruit samples were collected in Northern Portugal. The fruits were separated into pulp and peel. Each part of the fruit was cut into thin slices and freeze-dried.

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Lyophilizations were carried out using a Labconco 4.5 apparatus (Kansas City, MO). Quince jam samples were purchased on the Portuguese market.

*SPE cartridges*

The benzenesulfonic SCX Spe-ed SPE cartridges (200 mg; 3 mL) were obtained from Applied Separations (Allentown, USA).

*Extraction of free amino acids*

Each sample (*ca.* 1.5 g for freeze-dried quince pulps and peels and 5 g for quince jams) was thoroughly mixed with 3 × 25 mL of acid water (pH 2.2 with HCl 0.1 M), at room temperature, with magnetic stirring for 3 × 10 min. The extracts were gathered, filtered and passed through a SCX cartridge, previously conditioned with 10 ml of methanol and 10 mL of HCl 5 mM. The amino acids were eluted with a mixture of ammonia (4 M) and methanol (50:50 v/v) (3 × 500 µL). To each extract, 150 µL of L-*p*-chlorophenylalanine solution (10 µL/mL) (internal standard) were added. The obtained solutions were dried under $N_2$ stream and kept below 0°C until derivatization.

*Derivatization procedure*

The derivatization was achieved as previously reported.[3-6] Each dried residue was dissolved in 60 µL of water and 40 µL of ethanol/pyridine (4:1); 5 µL ECF were added and the sample was vortex mixed (3 - 5 s). Gas evolution (carbon dioxide) usually occurs. Five minutes later, 150 µL of dichloromethane and *ca.* 0.01 g of NaCl were added and the vial was thoroughly shaken for extraction of the derivatives into the organic layer. This phase was transferred into a 200 µL insert adjustable to the liquid sampler vials. About 1.5 µL was injected into the gas chromatographic system.

*GC analysis*

Separation of L-amino acids was achieved by gas chromatography, carried out with a Chrompack CP 9001 instrument (Chrompack, Middelburg, The Netherlands) equipped with a flame ionization detector (FID), and an automatic liquid sampler (CP-9050, Chrompack). The injector was kept at 250°C and the detector at 280°C. The GC was equipped with electronic pressure control allowing programmable gas pressure during the chromatographic run. Helium as carrier gas was used with the following pressure program: increase from initial 50 kPa to 70 kPa. A CP-Sil 19 CB (10 m × 0.25 mm i.d.) wcot fused-silica capillary column (Varian) was used with the following temperature program: increase from 140°C (1 min hold) to 280°C, at 40°C/min.

The compounds were identified by their retention times and chromatographic comparison with authentic standards. Quantification was based on the internal standard method using L-*p*-chlorophenylalanine.

## Results and Discussion

*Extraction method and SPE purification*

The extraction method was studied, based on published methodologies applied to other matrices[1,2,17,18] (fruit juices and jams, honeys and coffee beans), in order to obtain the highest recoveries and cleaner chromatograms. Acid water[1,2,17] (pH 2.2 with HCl 0.1 M), hydrochloric acid 0.1 M,[1,2] methanol 70%[1,2] and sulfosalicylic acid 2%[2,18] were tested on quince fruit (pulp and peel) and jam. Acid water (pH 2.2 with HCl 0.1 M) provided the best recoveries (data not shown).

Table 1 Concentration range of linearity and detection limits for amino acids

| Amino acid | Linearity/ µg mL$^{-1}$ | Detection limit/ µg mL$^{-1}$ |
|---|---|---|
| Alanine | 0 - 4.42 | 0.025 |
| Glycine | 0 - 4.22 | 0.017 |
| Valine | 0 - 4.10 | 0.010 |
| Leucine | 0 - 4.22 | 0.008 |
| Isoleucine | 0 - 4.10 | 0.012 |
| Proline | 0 - 3.80 | 0.007 |
| Threonine | 0 - 3.90 | 0.029 |
| Serine | 0 - 4.35 | 0.113 |
| Glutamic acid | 0 - 4.13 | 0.070 |
| Asparagine | 0 - 4.15 | 0.115 |
| Aspartic acid | 0 - 4.88 | 0.031 |
| Methionine | 0 - 4.27 | 0.012 |
| Hydroxyproline | 0 - 4.15 | 0.028 |
| Phenylalanine | 0 - 4.52 | 0.004 |
| Cysteine | 0 - 4.02 | 0.010 |
| Glutamine | 0 - 4.45 | 0.012 |
| Ornithine | 0 - 4.15 | 0.010 |
| Lysine | 0 - 3.95 | 0.007 |
| Histidine | 0 - 4.33 | 0.024 |
| Tyrosine | 0 - 4.42 | 0.004 |
| Tryptophan | 0 - 4.08 | 0.007 |

In order to remove interference compounds, a strong cation exchange (SCX) Solid-Phase Extraction (SPE) purification step was needed.

With the purpose of eliminating any disturbance compounds, after charging the sample solution to the cartridge, we have tried to wash the SCX cartridge with water and with acid water (pH 2.2). We have observed that better recoveries were obtained without washing the cartridge. It seems that some losses of amino acids occurred during the washing step.

The addition of internal standard was tested before and after the SCX step. When the internal standard was added after SPE purification, more accurate results were obtained.

*Derivatization procedure*

Since none of the amino acids is volatile enough for direct GC analysis, it is necessary to transform them into volatile derivatives. The chosen amino acids derivatization procedure with ethyl chloroformate is unique in rapidity, although one drawback is the inability to determine arginine, as already described by Husek.[3-6] Not all reactive groups in the amino acids are altered by action of the reagent. The imino group of arginine remains untouched, which is the reason for absorption of this amino acid derivative in the column.[3-6] For the determination of arginine, an additional reaction step would be necessary or, alternatively, its conversion into ornithine by arginase before derivatization.[18]

*GC analysis*

Separation of twenty-one amino acids was achieved by gas chromatography, with an analysis time of only 6 min, with good resolution, and low reagent and instrumentation costs.

*Analytical curves and detection limits*

Calibration curves were determined after subjecting standards to the same total procedure in order to compensate the losses during extraction, SPE clean-up and derivatization steps. Under the assay conditions described, a linear relationship between the

Table 2 Free amino acids composition of quince fruits (pulp and peel) and quince jams (μg $kg^{-1}$)[a] (quantification by internal standard technique)

| Amino acid | Fruit | | | | Jam | |
|---|---|---|---|---|---|---|
| | Pulp | | Peel | | | |
| | A | B | A | B | A | B |
| Alanine | 12.9(1.05) | 62.3(2.05) | 14.8(0.51) | 70.1(1.33) | 68.1(1.05) | 10.1(0.61) |
| Glycine | 67.8(3.18) | 177.9(5.64) | 13.6(0.34) | 355.0(6.32) | 65.3(1.92) | 45.2(2.57) |
| Valine | 13.8(0.64) | 47.9(1.21) | 13.6(0.45) | 63.0(1.21) | 19.5(0.70) | 6.4(0.16) |
| Leucine | 4.8(0.16) | 15.8(1.08) | 5.0(0.08) | 18.3(0.28) | 5.9(0.11) | 3.6(0.17) |
| Isoleucine | 13.7(0.26) | 70.2(2.97) | 15.4(0.70) | 125.4(2.20) | 23.4(0.45) | 12.5(0.48) |
| Proline | 5.0(0.21) | 15.6(0.59) | 5.8(0.25) | 24.9(0.43) | 6.2(0.12) | 3.1(0.02) |
| Threonine | 2.6(0.22) | 37.6(0.70) | 7.3(0.29) | 51.8(0.68) | 7.7(0.23) | 9.9(0.39) |
| Serine | 9.8(0.76) | 77.9(6.49) | 14.7(1.40) | 145.6(2.10) | 16.3(0.27) | 10.3(0.53) |
| Glutamic acid | 41.7(2.58) | 84.7(1.29) | 51.7(1.81) | 231.7(5.17) | 25.0(1.13) | 16.0(0.79) |
| Asparagine | 90.7(2.72) | 130.6(3.56) | 139.1(12.5) | 112.6(2.15) | 219.0(7.71) | 65.9(2.31) |
| Aspartic acid | 79.2(5.20) | 163.7(5.72) | 77.4(2.58) | 252.7(6.12) | 51.0(1.13) | 33.7(0.94) |
| Methionine | 0.4(0.03) | 1.4(0.02) | 2.2(0.06) | 0.4(0.02) | 0.4(0.02) | 0.3(0.01) |
| Hydroxyproline | 16.7(1.33) | 24.8(1.73) | 29.7(1.90) | 173.0(3.73) | 41.5(0.65) | 27.9(0.74) |
| Phenylalanine | 8.0(0.09) | 13.3(0.05) | 11.0(0.25) | 13.7(0.26) | 4.0(0.12) | 6.7(0.05) |
| Cysteine | 5.8(0.22) | 27.6(1.37) | 30.3(1.01) | 34.6(0.45) | 0.4(0.01) | 7.6(0.68) |
| Glutamine | 2.9(0.21) | 20.3(0.40) | 21.9(0.97) | 27.2(0.63) | 5.8(0.23) | 4.8(0.32) |
| Ornithine | 1.2(0.09) | 8.2(0.96) | 0.9(0.08) | 6.0(0.09) | 4.8(0.30) | 3.7(0.33) |
| Lysine | 12.5(0.33) | 38.0(2.05) | 22.8(0.94) | 49.6(0.98) | 14.9(0.35) | 8.9(0.38) |
| Histidine | 115.6(7.21) | 15.6(0.22) | 85.4(3.61) | 54.5(0.98) | 10.7(0.61) | 27.0(0.61) |
| Tyrosine | 1.1(0.06) | 3.4(0.11) | 0.5(0.04) | 5.6(0.09) | 1.9(0.10) | 1.1(0.06) |
| Tryptophan | 20.1(0.86) | 7.9(0.24) | 53.3(1.01) | 4.2(0.09) | 1.3(0.05) | 4.6(0.24) |

a. Values are expressed as mean (SD) of three determinations.

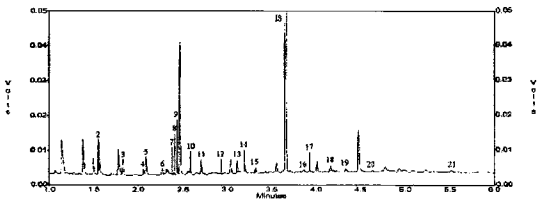

Fig. 1 Free amino acids profile of a quince pulp: (1) alanine, (2) glycine, (3) valine, (4) leucine, (5) isoleucine, (6) proline, (7) threonine, (8) serine, (9) glutamic acid, (10) asparagine, (11) aspartic acid, (12) methionine, (13) hydroxyproline, (14) phenylalanine, (15) cysteine, (IS) internal standard (L-*p*-chlorophenylalanine), (16) glutamine, (17) ornithine, (18) lysine, (19) histidine, (20) tyrosine and (21) tryptophan.

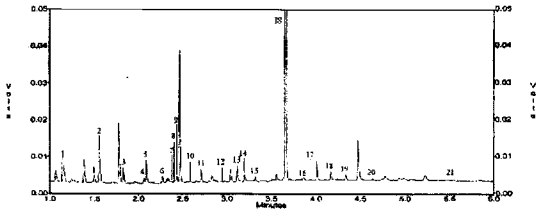

Fig. 2 Free amino acids profile of a quince peel. (1) - (12) are the same as in Fig. 1.

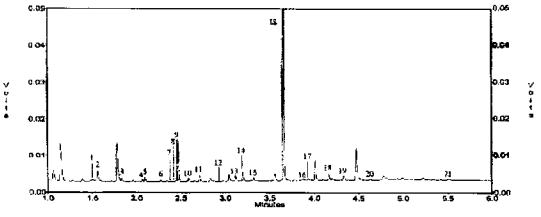

Fig. 3 Free amino acids profile of a quince jam. (1) - (12) are the same as in Fig. 1.

concentration of amino acids and the FID response was obtained in the tested range. The correlation coefficient for the standard curves invariably exceeded 0.99, for all the compounds.

The detection limit values were calculated as the concentration corresponding to three times the standard deviation of the background noise, and the values obtained ranged from 0.004 to 0.115 μg/ml. (Table 1).

*Validation of the method*

This method was specially developed for the determination of amino acids in quince jams, fruit products with high amounts of sugar and other interfering compounds, some of them produced during thermal processing. Since quince pulps and peels are less complex matrices, the developed technique was also applied to them (we only tested the accuracy of the procedure for these matrices).

The free amino acids from quince fruit (pulp and peel) and quince jam samples were analyzed by the proposed technique in order to validate this procedure and to assess its applicability to the routine free amino acid analysis of these food products (Table 2). Typical chromatograms obtained with quince pulp, peel and jam samples are represented in Figs. 1, 2 and 3, respectively. The retention times (RT) obtained for amino acids

Table 3 Retention times of the amino acids ($n$ = 3)

| Amino acid | Retention time[a]/min | SD/min |
|---|---|---|
| Alanine | 1.50 | 0.001 |
| Glycine | 1.59 | 0.001 |
| Valine | 1.83 | 0.001 |
| Leucine | 2.06 | 0.001 |
| Isoleucine | 2.09 | 0.001 |
| Proline | 2.27 | 0.002 |
| Threonine | 2.34 | 0.001 |
| Serine | 2.37 | 0.001 |
| Glutamic acid | 2.44 | 0.001 |
| Asparagine | 2.57 | 0.002 |
| Aspartic acid | 2.72 | 0.002 |
| Methionine | 2.93 | 0.003 |
| Hydroxyproline | 3.12 | 0.003 |
| Phenylalanine | 3.20 | 0.002 |
| Cysteine | 3.29 | 0.002 |
| Internal standard | 3.66 | 0.002 |
| Glutamine | 3.87 | 0.001 |
| Ornithine | 3.94 | 0.003 |
| Lysine | 4.17 | 0.002 |
| Histidine | 4.33 | 0.004 |
| Tyrosine | 4.64 | 0.003 |
| Tryptophan | 5.52 | 0.004 |

a. Mean of the retention times; SD, standard deviation.

Table 4 Evaluation of the analytical method precision ($n$ = 6) (quantification by internal standard technique)

| Amino acid | SD/μg kg$^{-1}$ | CV, % |
|---|---|---|
| Alanine | 0.37 | 0.72 |
| Glycine | 0.21 | 0.52 |
| Valine | 0.15 | 1.47 |
| Leucine | 0.03 | 0.65 |
| Isoleucine | 0.13 | 0.85 |
| Proline | 0.03 | 0.97 |
| Threonine | 0.04 | 0.98 |
| Serine | 0.18 | 1.31 |
| Glutamic acid | 0.03 | 0.25 |
| Asparagine | 2.34 | 1.14 |
| Aspartic acid | 0.66 | 1.67 |
| Methionine | 0.01 | 5.22 |
| Hydroxyproline | 0.53 | 1.58 |
| Phenylalanine | 0.02 | 0.46 |
| Cysteine | 0.01 | 0.42 |
| Glutamine | 0.03 | 0.98 |
| Ornithine | 0.04 | 1.67 |
| Lysine | 0.02 | 0.18 |
| Histidine | 0.15 | 1.93 |
| Tyrosine | 0.01 | 1.76 |
| Tryptophan | 0.01 | 0.91 |

SD, standard deviation; CV, coefficient of variation.

are shown in Table 3.

The precision of the analytical method was evaluated by measuring the peak chromatographic area of amino acids six times on the same quince jam sample. The analytical method is precise, once the coefficients of variation of free amino acids were low (between 0.25 and 5.22%) (Table 4).

The reproducibility of the method was evaluated by the calculation of the coefficient of variation for each free amino acid of six repeated extractions of the same quince jam sample. Considering that we are dealing with food matrices with low free amino acids content, in a general way, we found that the

Table 5 Evaluation of the analytical method reproducibility ($n$ = 6) (quantification by internal standard technique)

| Amino acid | SD/μg kg$^{-1}$ | CV, % |
|---|---|---|
| Alanine | 1.65 | 2.42 |
| Glycine | 2.32 | 3.55 |
| Valine | 0.73 | 3.74 |
| Leucine | 0.15 | 2.53 |
| Isoleucine | 0.55 | 2.34 |
| Proline | 0.29 | 4.75 |
| Threonine | 0.28 | 3.61 |
| Serine | 0.37 | 2.24 |
| Glutamic acid | 1.51 | 6.02 |
| Asparagine | 8.74 | 3.99 |
| Aspartic acid | 1.30 | 2.54 |
| Methionine | 0.04 | 10.30 |
| Hydroxyproline | 0.86 | 2.07 |
| Phenylalanine | 0.22 | 5.40 |
| Cysteine | 0.02 | 4.33 |
| Glutamine | 0.30 | 5.20 |
| Ornithine | 0.35 | 7.40 |
| Lysine | 0.52 | 3.47 |
| Histidine | 0.68 | 6.33 |
| Tyrosine | 0.13 | 6.93 |
| Tryptophan | 0.07 | 5.17 |

SD, standard deviation; CV, coefficient of variation.

coefficient of variation values were low (Table 5) and the method is reproducible. For the cases where coefficients were higher, they, generally, correspond to the amino acids that were present in very low amounts, such as methionine. However, no linear relation between the content and the coefficient of variation was observed, but in a general way coefficients higher than 6.5 were found for amino acids in amounts below 5 μg/kg.

The accuracy (% recovery) of the procedure was evaluated in triplicate using the same quince fruit (pulp and peel) and quince jam samples spiked with known standard amounts. Generally, the recovery values were high (Tables 6, 7 and 8), which demonstrates the effectiveness of the extraction and the accuracy of the method.

In conclusion, the proposed GC/FID procedure for free amino acid profile determination is simple, sensitive, reproducible and accurate. This method has the main advantage of being very rapid, especially in terms of derivatization process and run time, which makes it suitable for routine analysis of amino acids in quality control determinations of quince jam and fruit. Its only limitation is the inability to determine arginine.

In future studies, we will apply this method in the determination of free amino acid profiles of quince jams of several homemade and commercially available samples, quince fruits (pulps and peels) from several geographical origins of Portugal, during three consecutive years, in order to test if this pattern can be useful in the evaluation of quince jam authenticity.

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Table 6 Recoveries of free amino acids from a spiked quince jam sample[a]

| Amino acid | Present/ μg kg$^{-1}$ | Added/ μg kg$^{-1}$ | Found/ μg kg$^{-1}$ | SD/ μg kg$^{-1}$ | CV, % | Recovery, % |
|---|---|---|---|---|---|---|
| Alanine | 68.1 | 2.6 | 71.5 | 1.68 | 2.35 | 101.1 |
| | | 5.1 | 75.8 | 1.66 | 2.19 | 103.6 |
| | | 10.6 | 86.8 | 2.13 | 2.46 | 110.3 |
| Glycine | 65.3 | 2.5 | 74.3 | 2.53 | 3.41 | 109.5 |
| | | 5.0 | 71.4 | 2.25 | 3.16 | 101.4 |
| | | 10.1 | 77.5 | 3.16 | 4.08 | 102.7 |
| Valine | 19.5 | 2.4 | 24.3 | 0.71 | 2.90 | 110.8 |
| | | 4.9 | 29.2 | 0.88 | 3.01 | 119.5 |
| | | 9.8 | 33.4 | 0.66 | 1.99 | 113.7 |
| Leucine | 5.9 | 2.5 | 8.7 | 0.23 | 2.68 | 103.9 |
| | | 5.0 | 12.2 | 0.31 | 2.54 | 111.6 |
| | | 10.1 | 17.3 | 0.52 | 3.00 | 108.5 |
| Isoleucine | 23.4 | 2.4 | 25.5 | 0.79 | 3.10 | 98.6 |
| | | 4.9 | 30.3 | 0.90 | 2.96 | 107.0 |
| | | 9.8 | 34.8 | 0.91 | 2.61 | 104.8 |
| Proline | 6.2 | 2.3 | 6.7 | 0.28 | 4.18 | 79.3 |
| | | 4.5 | 9.7 | 0.39 | 3.98 | 91.0 |
| | | 9.1 | 12.6 | 0.45 | 3.57 | 82.3 |
| Threonine | 7.7 | 2.2 | 9.4 | 0.29 | 3.12 | 94.7 |
| | | 9.3 | 12.5 | 0.51 | 4.07 | 73.4 |
| | | 18.7 | 22.7 | 0.68 | 2.99 | 86.1 |
| Serine | 16.3 | 2.6 | 19.4 | 0.34 | 1.76 | 102.4 |
| | | 5.2 | 24.9 | 0.50 | 1.99 | 116.0 |
| | | 10.4 | 29.6 | 0.76 | 2.56 | 110.7 |
| Glutamic acid | 25.0 | 4.9 | 32.3 | 1.11 | 3.44 | 107.9 |
| | | 9.9 | 35.2 | 1.33 | 3.78 | 100.9 |
| | | 19.3 | 41.0 | 1.23 | 3.00 | 92.5 |
| Asparagine | 219.0 | 2.5 | 263.2 | 6.76 | 2.57 | 118.8 |
| | | 9.9 | 272.8 | 7.30 | 2.68 | 119.2 |
| | | 19.9 | 253.2 | 5.91 | 2.33 | 106.0 |
| Aspartic acid | 51.0 | 2.9 | 52.6 | 1.53 | 2.90 | 97.6 |
| | | 11.7 | 55.8 | 1.42 | 2.55 | 89.0 |
| | | 22.8 | 71.9 | 1.52 | 2.11 | 97.4 |
| Methionine | 0.4 | 2.4 | 2.5 | 0.13 | 5.33 | 89.3 |
| | | 4.9 | 3.9 | 0.22 | 5.67 | 73.5 |
| | | 10.2 | 8.0 | 0.41 | 5.12 | 75.1 |
| Hydroxyproline | 41.5 | 2.4 | 44.0 | 0.44 | 0.99 | 100.4 |
| | | 9.9 | 46.4 | 0.93 | 2.00 | 90.2 |
| | | 19.4 | 61.9 | 1.67 | 2.70 | 101.7 |
| Phenylalanine | 4.0 | 5.4 | 8.6 | 0.26 | 3.02 | 91.1 |
| | | 10.8 | 16.0 | 0.32 | 1.99 | 107.9 |
| | | 21.7 | 27.6 | 0.76 | 2.74 | 107.4 |
| Cysteine | 0.4 | 2.3 | 2.4 | 0.06 | 2.68 | 89.3 |
| | | 4.8 | 4.4 | 0.16 | 3.67 | 83.9 |
| | | 9.6 | 8.0 | 0.32 | 4.00 | 79.6 |
| Glutamine | 5.8 | 2.5 | 7.6 | 0.22 | 2.81 | 91.5 |
| | | 5.1 | 10.1 | 0.21 | 2.10 | 92.4 |
| | | 21.4 | 26.2 | 0.96 | 3.67 | 96.6 |
| Ornithine | 4.8 | 4.8 | 8.5 | 0.24 | 2.89 | 89.3 |
| | | 9.9 | 12.6 | 0.32 | 2.56 | 85.9 |
| | | 19.9 | 24.3 | 0.48 | 1.99 | 98.6 |
| Lysine | 14.9 | 4.5 | 18.7 | 0.56 | 3.00 | 96.5 |
| | | 9.5 | 23.3 | 0.65 | 2.79 | 95.8 |
| | | 18.9 | 35.8 | 1.09 | 3.06 | 105.8 |
| Histidine | 10.7 | 2.6 | 13.8 | 0.41 | 2.95 | 103.6 |
| | | 10.4 | 24.7 | 0.56 | 2.26 | 117.0 |
| | | 20.8 | 33.4 | 0.52 | 1.56 | 105.9 |
| Tyrosine | 1.9 | 2.6 | 4.4 | 0.16 | 3.66 | 98.2 |
| | | 5.3 | 7.3 | 0.21 | 2.88 | 101.5 |
| | | 10.6 | 11.6 | 0.09 | 0.79 | 93.5 |
| Tryptophan | 1.3 | 2.4 | 3.7 | 0.17 | 4.61 | 100.3 |
| | | 4.9 | 7.1 | 0.22 | 3.10 | 114.6 |
| | | 9.8 | 12.0 | 0.22 | 1.87 | 108.5 |

a. Mean value found for three assays for each studied concentration; SD, standard deviation; CV, coefficient of variation.

Table 7 Recoveries of free amino acids from a spiked quince pulp[a]

| Amino acid | Present/ μg kg$^{-1}$ | Added/ μg kg$^{-1}$ | Found/ μg kg$^{-1}$ | SD/ μg kg$^{-1}$ | CV, % | Recovery, % |
|---|---|---|---|---|---|---|
| Alanine | 12.9 | 10.8 | 24.1 | 0.55 | 2.28 | 101.6 |
| Glycine | 67.8 | 10.4 | 81.6 | 2.57 | 3.15 | 104.4 |
| Valine | 13.8 | 10.1 | 25.4 | 0.54 | 2.11 | 106.7 |
| Leucine | 4.8 | 10.4 | 15.4 | 0.49 | 3.21 | 101.5 |
| Isoleucine | 13.7 | 10.1 | 25.6 | 0.66 | 2.60 | 107.5 |
| Proline | 5.0 | 9.2 | 12.8 | 0.46 | 3.61 | 90.4 |
| Threonine | 2.6 | 9.6 | 9.2 | 0.27 | 2.89 | 75.6 |
| Serine | 9.8 | 10.7 | 18.3 | 0.41 | 2.25 | 89.6 |
| Glutamic acid | 41.7 | 10.0 | 55.6 | 1.36 | 2.44 | 107.5 |
| Asparagine | 90.7 | 10.2 | 104.4 | 2.19 | 2.10 | 103.5 |
| Aspartic acid | 79.2 | 12.0 | 90.7 | 2.33 | 2.57 | 99.4 |
| Methionine | 0.4 | 10.3 | 8.6 | 0.41 | 4.75 | 80.5 |
| Hydroxyproline | 16.7 | 10.2 | 26.8 | 0.31 | 1.16 | 99.6 |
| Phenylalanine | 8.0 | 10.9 | 16.8 | 0.45 | 2.69 | 88.8 |
| Cysteine | 5.8 | 9.9 | 12.6 | 0.50 | 3.98 | 80.4 |
| Glutamine | 2.9 | 10.9 | 14.1 | 0.36 | 2.56 | 102.2 |
| Ornithine | 1.2 | 10.2 | 10.2 | 0.24 | 2.33 | 89.4 |
| Lysine | 12.5 | 9.6 | 23.7 | 0.64 | 2.71 | 107.8 |
| Histidine | 115.6 | 10.6 | 117.9 | 2.00 | 1.70 | 93.4 |
| Tyrosine | 1.1 | 10.8 | 12.5 | 0.25 | 1.98 | 105.1 |
| Tryptophan | 20.1 | 10.0 | 31.0 | 1.12 | 3.62 | 102.9 |

a. Mean value found for three assays for each studied concentration; SD, standard deviation; CV, coefficient of variation.

Table 8 Recoveries of free amino acids from a spiked quince pulp[a]

| Amino acid | Present/ μg kg$^{-1}$ | Added/ μg kg$^{-1}$ | Found/ μg kg$^{-1}$ | SD/ μg kg$^{-1}$ | CV, % | Recovery, % |
|---|---|---|---|---|---|---|
| Alanine | 14.8 | 12.1 | 27.8 | 0.65 | 2.33 | 103.5 |
| Glycine | 13.6 | 11.3 | 24.7 | 0.86 | 3.48 | 99.2 |
| Valine | 13.6 | 11.2 | 26.4 | 0.52 | 1.98 | 106.4 |
| Leucine | 5.0 | 11.5 | 16.5 | 0.53 | 3.22 | 99.9 |
| Isoleucine | 15.4 | 11.2 | 26.9 | 0.45 | 1.67 | 101.3 |
| Proline | 5.8 | 10.2 | 13.1 | 0.51 | 3.91 | 81.8 |
| Threonine | 7.3 | 10.7 | 13.6 | 0.42 | 3.08 | 75.9 |
| Serine | 14.7 | 11.9 | 25.6 | 0.70 | 2.71 | 96.2 |
| Glutamic acid | 51.7 | 11.1 | 65.9 | 1.91 | 2.90 | 104.9 |
| Asparagine | 139.1 | 11.1 | 149.2 | 3.23 | 2.16 | 99.3 |
| Aspartic acid | 77.4 | 13.3 | 80.5 | 1.34 | 1.67 | 88.7 |
| Methionine | 2.2 | 11.4 | 13.3 | 0.58 | 4.36 | 97.3 |
| Hydroxyproline | 29.7 | 11.1 | 36.5 | 0.72 | 1.96 | 89.3 |
| Phenylalanine | 11.0 | 12.3 | 23.0 | 0.50 | 2.18 | 98.5 |
| Cysteine | 30.3 | 10.8 | 32.5 | 1.30 | 4.00 | 79.1 |
| Glutamine | 21.9 | 11.9 | 31.9 | 0.74 | 2.32 | 94.2 |
| Ornithine | 0.9 | 11.3 | 11.6 | 0.30 | 2.57 | 95.1 |
| Lysine | 22.8 | 10.6 | 33.2 | 0.85 | 2.56 | 99.2 |
| Histidine | 85.4 | 11.8 | 111.3 | 2.17 | 1.95 | 114.5 |
| Tyrosine | 0.5 | 11.8 | 11.3 | 0.23 | 2.07 | 91.3 |
| Tryptophan | 53.3 | 11.2 | 63.1 | 2.18 | 3.45 | 97.8 |

a. Mean value found for three assays for each studied concentration; SD, standard deviation; CV, coefficient of variation.

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# Free Amino Acid Composition of Quince (*Cydonia oblonga* Miller) Fruit (Pulp and Peel) and Jam

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Twenty-one free amino acids present in several samples of quince fruit (pulp and peel) and quince jam (homemade and industrially manufactured) were analyzed by GC/FID. The analyses showed some differences between quince pulps and peels. Generally, the highest content in total free amino acids and in glycine was found in peels. As a general rule, the three major free amino acids detected in pulps were aspartic acid, asparagine, and hydroxyproline. For quince peels, usually, the three most abundant amino acids were glycine, aspartic acid, and asparagine. Similarly, for quince jams the most important free amino acids were aspartic acid, asparagine, and glycine or hydroxyproline. This study suggests that the free amino acid analysis can be useful for the evaluation of quince jam authenticity. It seems that glycine percentage can be used for the detection of quince peel addition while high alanine content can be related to pear addition.

KEYWORDS: *Cydonia oblonga* Miller; quince fruit; quince jam; GC/FID; free amino acids

## INTRODUCTION

Besides proteins, plants and fruits possess amino acids in their free form, which mostly show a typical pattern (*1*). Determination of this pattern provides some information about possible adulteration or falsification of their derivatives, namely, fruit juices, wines, and jams (*1*, *2*).

Amino acids, a class of biologically active compounds present in food and beverages, are important for human nutrition and affect the quality of foods including taste, aroma, and color (*1*). Among different substances that constitute fruits and vegetables, amino acids are becoming increasingly important and, for various reasons, their analytical determination is becoming more necessary (*3*). First, the concentration of amino acids in the fruit varies significantly as a result of metabolic changes during growth and ripening, and this can be exploited to determine the optimum ripening time (*3*). Second, amino acid profiles vary from one species to another and among fruits of the same type but with different origin, so they can probably be used to characterize fruit products (*3*, *4*). Finally, amino acids can influence the quality of fruit-derived products because they take an active part in the Maillard reaction and in browning processes after the enzymatic oxidation of polyphenols, which determine the sensorial quality of such products like juices and jams (*3*).

Quince is the fruit of a deciduous tree of the *Rosaceae* family, *Cydonia oblonga* Miller. Because of its hardness, acidity, and astringency, it is not edible unprocessed; nevertheless, it is often used to prepare jam. Quince jam, either homemade or industrially manufactured, is obtained by boiling a mixture of sugar and quince puree until convenient consistency is obtained (usually to reach 65−72 °Brix). When quince production is scarce, industry manufacturers are tempted to adulterate quince jam by adding apple (*Malus communis* Lamk) and/or pear (*Pirus communis* Lin.) because of their low cost and similar texture and rheological properties. Once the stronger odor of quince masks the sweet flavors of both fruits, sensory evaluation cannot be used to detect their presence (*5*).

Few chemical studies have been developed for quince fruit and its derivatives. In 1979 and 1986, the volatile constituents of quince fruit were analyzed by GC/MS (*6*, *7*). Later, the usefulness of phenolic compounds in the determination of genuineness of quince puree (*8*), jam (*9*, *10*), and jelly (*5*) has been reported. Glucosides of procyanidin polymers have been previously identified in this fruit (*11*, *12*). Recently, it was possible to discriminate quince pulp and peel by the analysis of phenolic compounds in quince fruit (*13*). In 2002, an HPLC/UV method was developed for the determination of organic acids in quince fruit and its jam (*14*). More recently, a GC/FID method was developed for the determination of free amino acids in quince fruit and jam (*15*). The referred method was then applied to the analysis of samples of quince fruit (pulp and peel) from seven different geographic origins from Portugal, as well as 20 commercial quince jam samples (4 homemade and 16 industrially manufactured).

## MATERIALS AND METHODS

**Samples.** Healthy quince fruit samples were collected in different places in Northern (Amarante, Baião, Vila Real, and Bragança) and

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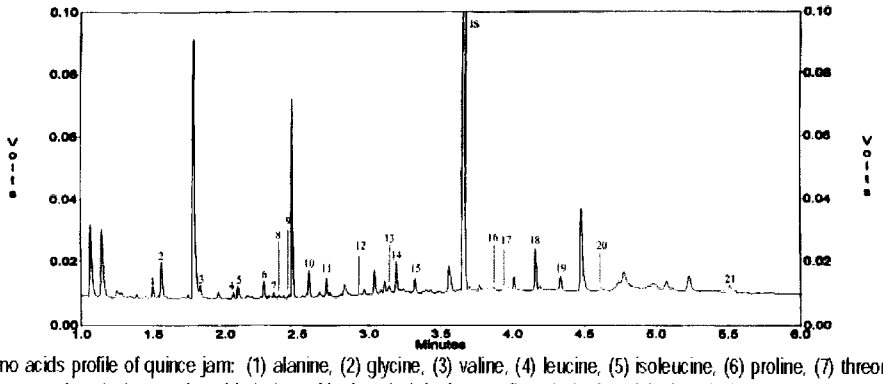


**Figure 1.** Free amino acids profile of quince jam: (1) alanine, (2) glycine, (3) valine, (4) leucine, (5) isoleucine, (6) proline, (7) threonine, (8) serine, (9) glutamic acid, (10) asparagine, (11) aspartic acid, (12) methionine, (13) hydroxyproline, (14) phenylalanine, (15) cysteine, (IS) internal standard (L-*p*-chlorophenylalanine), (16) glutamine, (17) ornithine, (18) lysine, (19) histidine, (20) tyrosine, and (21) tryptophan.

**Table 1.** Retention Times of the Amino Acids ($n = 3$)

| code | amino acid | retention time[a] (min) | SD (min) |
|---|---|---|---|
| 1 | alanine | 1.50 | 0.001 |
| 2 | glycine | 1.59 | 0.001 |
| 3 | valine | 1.83 | 0.001 |
| 4 | leucine | 2.06 | 0.001 |
| 5 | isoleucine | 2.09 | 0.001 |
| 6 | proline | 2.27 | 0.002 |
| 7 | threonine | 2.34 | 0.001 |
| 8 | serine | 2.37 | 0.001 |
| 9 | glutamic acid | 2.44 | 0.001 |
| 10 | asparagine | 2.57 | 0.002 |
| 11 | aspartic acid | 2.72 | 0.002 |
| 12 | methionine | 2.93 | 0.003 |
| 13 | hydroxyproline | 3.12 | 0.003 |
| 14 | phenylalanine | 3.20 | 0.002 |
| 15 | cysteine | 3.29 | 0.002 |
| IS | internal standard | 3.66 | 0.002 |
| 16 | glutamine | 3.87 | 0.001 |
| 17 | ornithine | 3.94 | 0.003 |
| 18 | lysine | 4.17 | 0.002 |
| 19 | histidine | 4.33 | 0.004 |
| 20 | tyrosine | 4.64 | 0.003 |
| 21 | tryptophan | 5.52 | 0.004 |

[a] Mean of the retention times; SD, standard deviation.

Central Portugal (Viseu, Pinhel, and Covilhã). All fruits were separated into pulp and peel. Each part of the fruit was cut into thin slices and freeze-dried. Lyophilization was carried out using a Labconco 4.5 apparatus (Kansas City, MO).

Twenty commercial quince jam samples, including 4 homemade (samples A−D) and 16 industrially manufactured (samples E−T), randomly purchased on the Portuguese market were assayed.

**Standards.** The 21 L-amino acids and the internal standard (L-*p*-chlorophenylalanine) were all from Sigma (St. Louis, MO). Ethyl chloroformate (ECF) was from Aldrich (Steinheim, Germany), and pyridine was from Fluka (Neu-Ulm, Germany). All other chemicals were analytical grade from several suppliers.

**Solid-Phase Extraction (SPE) Cartridges.** The benzenesulfonic SCX Spe-ed SPE cartridges (200 mg, 3 mL) were obtained from Applied Separations (Allentown, PA).

**Extraction of Free Amino Acids.** The extraction was achieved as previously reported (*15*). Each sample (about 1.5 g for freeze-dried quince pulps and peels and 5 g for quince jams) was thoroughly mixed with 3 × 25 mL of acid water (pH 2.2 with 0.1 M HCl) at room temperature with magnetic stirring for 3 × 10 min. The extracts were gathered, filtered, and passed through a SCX cartridge previously conditioned with 10 mL of methanol and 10 mL of 5 mM HCl. The amino acids were eluted with a mixture of ammonia (4 M) and methanol

**Table 2.** Free Amino Acids Composition of Quince Pulps from Several Geographic Locations from Portugal (μg/kg) (Quantification by Internal Standard Technique)[a]

| | geographic origin | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Amarante | | Baião | | Vila Real | | Bragança | | Covilhã | | Viseu | | Pinhel | |
| amino acids | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD |
| alanine | 12.9 | 1.05 | 62.3 | 2.05 | 61.2 | 2.33 | 16.9 | 0.51 | 14.2 | 0.55 | 12.5 | 0.45 | 48.7 | 1.39 |
| glycine | 67.8 | 3.18 | 177.9 | 5.64 | 40.3 | 1.31 | 144.6 | 5.69 | 4.8 | 0.21 | 6.4 | 0.29 | 47.0 | 2.87 |
| valine | 13.8 | 0.64 | 47.9 | 1.21 | 13.3 | 0.60 | 19.0 | 0.62 | 4.5 | 0.16 | 5.8 | 0.25 | 21.1 | 1.09 |
| leucine | 4.8 | 0.16 | 15.8 | 1.08 | 3.8 | 0.15 | 4.9 | 0.31 | 2.1 | 0.13 | 1.3 | 0.05 | 6.7 | 0.11 |
| isoleucine | 13.7 | 0.26 | 70.2 | 2.97 | 14.2 | 0.06 | 12.8 | 0.62 | 11.6 | 0.72 | 6.1 | 0.18 | 21.1 | 0.57 |
| proline | 5.0 | 0.21 | 15.6 | 0.59 | 18.7 | 0.47 | 11.2 | 0.38 | 3.4 | 0.16 | 3.3 | 0.14 | 8.8 | 0.45 |
| threonine | 2.6 | 0.22 | 37.6 | 0.70 | 9.0 | 0.37 | 10.1 | 0.52 | 14.7 | 0.82 | 7.3 | 0.05 | 10.2 | 0.32 |
| serine | 9.8 | 0.76 | 77.9 | 6.49 | 39.4 | 2.26 | 19.2 | 0.22 | 51.6 | 0.69 | 14.5 | 0.22 | 33.1 | 1.64 |
| glutamic acid | 41.7 | 2.58 | 84.7 | 1.29 | 43.1 | 1.01 | 47.1 | 0.48 | 54.8 | 2.41 | 11.4 | 0.69 | 166.8 | 11.65 |
| asparagine | 90.7 | 2.72 | 130.6 | 3.56 | 307.1 | 6.33 | 188.7 | 5.86 | 54.7 | 0.37 | 40.3 | 2.12 | 121.4 | 3.55 |
| aspartic acid | 79.2 | 5.20 | 163.7 | 5.72 | 85.0 | 3.18 | 242.8 | 2.28 | 186.9 | 10.16 | 111.2 | 0.45 | 460.8 | 15.45 |
| methionine | 0.4 | 0.03 | 1.4 | 0.02 | 2.8 | 0.03 | 1.8 | 0.10 | 4.7 | 0.18 | 1.1 | 0.07 | 4.7 | 0.21 |
| hydroxyproline | 16.7 | 1.33 | 24.8 | 1.73 | 74.4 | 4.05 | 205.7 | 4.15 | 75.2 | 2.85 | 53.2 | 2.64 | 264.6 | 5.10 |
| phenylalanine | 8.0 | 0.09 | 13.3 | 0.05 | 4.9 | 0.06 | 3.0 | 0.17 | 1.2 | 0.02 | 0.6 | 0.02 | 14.2 | 0.52 |
| cysteine | 5.8 | 0.22 | 27.6 | 1.37 | 31.9 | 0.92 | 24.0 | 0.92 | 69.0 | 3.90 | 16.6 | 0.85 | 56.3 | 3.02 |
| glutamine | 2.9 | 0.21 | 20.3 | 0.40 | 2.4 | 0.18 | 35.0 | 2.32 | 6.7 | 0.15 | 8.2 | 0.16 | 28.8 | 0.30 |
| ornithine | 1.2 | 0.09 | 8.2 | 0.96 | 0.6 | 0.02 | 1.6 | 0.12 | 0.8 | 0.04 | 1.3 | 0.01 | 1.3 | 0.02 |
| lysine | 12.5 | 0.33 | 38.0 | 2.05 | 9.3 | 0.24 | 21.4 | 0.85 | 9.7 | 0.33 | 6.3 | 0.26 | 12.2 | 0.71 |
| histidine | 115.6 | 7.21 | 15.6 | 0.22 | 5.8 | 0.24 | 26.5 | 0.51 | 8.0 | 0.43 | 6.5 | 0.14 | 15.0 | 1.29 |
| tyrosine | 1.1 | 0.06 | 3.4 | 0.11 | 0.4 | 0.01 | 0.4 | 0.01 | 0.4 | 0.01 | 0.2 | 0.01 | 1.2 | 0.04 |
| tryptophan | 20.1 | 0.86 | 7.9 | 0.24 | 4.2 | 0.16 | 18.7 | 0.89 | 0.6 | 0.01 | 1.9 | 0.08 | 12.5 | 0.40 |
| Σ | 526.2 | | 1044.6 | | 771.9 | | 1055.4 | | 579.6 | | 315.9 | | 1356.6 | |

[a] Values are expressed as the mean of three determinations; SD, standard deviation; Σ, sum of the determined free amino acids.

**Table 3.** Free Amino Acids Composition of Quince Peels from Several Geographic Locations from Portugal (μg/kg) (Quantification by Internal Standard Technique)[a]

| | geographic origin | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Amarante | | Baião | | Vila Real | | Bragança | | Covilhã | | Viseu | | Pinhel | |
| amino acids | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD |
| alanine | 14.8 | 0.51 | 70.1 | 1.33 | 36.5 | 0.41 | 22.7 | 1.21 | 10.8 | 0.30 | 33.4 | 1.05 | 31.1 | 1.16 |
| glycine | 13.6 | 0.34 | 355.0 | 6.32 | 99.3 | 7.43 | 353.5 | 20.92 | 314.9 | 18.63 | 238.9 | 5.28 | 148.3 | 8.07 |
| valine | 13.6 | 0.45 | 63.0 | 1.21 | 6.4 | 0.27 | 27.3 | 0.93 | 12.4 | 0.43 | 24.4 | 0.64 | 16.0 | 0.68 |
| leucine | 5.0 | 0.08 | 18.3 | 0.28 | 1.3 | 0.03 | 5.7 | 0.22 | 1.4 | 0.07 | 2.2 | 0.11 | 3.0 | 0.04 |
| isoleucine | 15.4 | 0.70 | 125.4 | 2.20 | 22.3 | 0.63 | 55.3 | 1.74 | 35.2 | 0.78 | 24.8 | 0.60 | 19.0 | 0.66 |
| proline | 5.8 | 0.25 | 24.9 | 0.43 | 9.5 | 0.42 | 14.0 | 0.31 | 3.4 | 0.06 | 13.3 | 0.37 | 6.1 | 0.17 |
| threonine | 7.3 | 0.29 | 51.8 | 0.68 | 14.5 | 0.62 | 12.9 | 0.88 | 8.9 | 0.51 | 4.8 | 0.19 | 8.1 | 0.30 |
| serine | 14.7 | 1.40 | 145.6 | 2.10 | 28.1 | 1.55 | 29.5 | 1.97 | 21.1 | 1.28 | 30.8 | 1.30 | 38.2 | 0.52 |
| glutamic acid | 51.7 | 1.81 | 231.7 | 5.17 | 37.2 | 0.17 | 115.1 | 4.91 | 22.4 | 0.69 | 34.8 | 0.84 | 97.0 | 3.17 |
| asparagine | 139.1 | 12.46 | 112.6 | 2.15 | 101.9 | 3.65 | 207.0 | 4.26 | 40.4 | 0.63 | 177.6 | 6.32 | 72.8 | 1.95 |
| aspartic acid | 77.4 | 2.58 | 252.7 | 6.12 | 61.4 | 2.50 | 320.7 | 15.96 | 103.8 | 5.65 | 242.6 | 10.56 | 329.7 | 2.05 |
| methionine | 2.2 | 0.06 | 0.4 | 0.02 | 4.0 | 0.25 | 1.9 | 0.02 | 2.5 | 0.12 | 1.4 | 0.12 | 0.25 | 0.03 |
| hydroxyproline | 29.7 | 1.90 | 173.0 | 3.73 | 14.9 | 0.08 | 204.3 | 13.35 | 76.8 | 3.67 | 79.0 | 2.27 | 33.1 | 0.64 |
| phenylalanine | 11.0 | 0.25 | 13.7 | 0.26 | 2.9 | 0.10 | 6.0 | 0.39 | 0.8 | 0.05 | 1.1 | 0.04 | 4.2 | 0.17 |
| cysteine | 30.3 | 1.01 | 34.6 | 0.45 | 51.2 | 1.24 | 41.5 | 1.82 | 33.9 | 2.51 | 12.3 | 0.16 | 16.8 | 0.73 |
| glutamine | 21.9 | 0.97 | 27.2 | 0.63 | 2.8 | 0.01 | 12.2 | 0.31 | 1.5 | 0.04 | 19.4 | 0.56 | 1.7 | 0.12 |
| ornithine | 0.9 | 0.08 | 6.0 | 0.09 | 0.9 | 0.03 | 1.0 | 0.01 | 0.6 | 0.04 | 7.6 | 0.16 | 2.0 | 0.03 |
| lysine | 22.8 | 0.94 | 49.6 | 0.98 | 10.7 | 0.50 | 27.2 | 0.96 | 5.8 | 0.44 | 10.3 | 0.14 | 8.3 | 0.28 |
| histidine | 85.4 | 3.61 | 54.5 | 0.98 | 4.8 | 0.24 | 55.5 | 1.14 | 2.7 | 0.04 | 51.0 | 1.80 | 34.5 | 0.91 |
| tyrosine | 0.5 | 0.04 | 5.6 | 0.09 | 0.5 | 0.04 | 2.5 | 0.16 | 0.3 | 0.01 | 1.0 | 0.05 | 0.5 | 0.01 |
| tryptophan | 53.3 | 1.01 | 4.2 | 0.09 | 0.5 | 0.02 | 14.1 | 0.85 | 0.5 | 0.01 | 6.5 | 0.36 | 3.7 | 0.04 |
| Σ | 616.3 | | 1819.9 | | 511.7 | | 1529.9 | | 700.1 | | 1017.3 | | 874.1 | |

[a] Values are expressed as the mean of three determinations; SD, standard deviation; Σ, sum of the determined free amino acids.

(50:50 v/v) (3 × 500 μL). To each extract, an amount of 150 μL of L-*p*-chlorophenylalanine solution (10 μL/mL) (internal standard) was added. The obtained solutions were dried under $N_2$ stream and kept below 0 °C until derivatization.

**Derivatization Procedure.** The derivatization was achieved as previously reported (*15*−*19*). Each dried residue was dissolved in 60 μL of water and 40 μL of ethanol/pyridine (4:1), an amount of 5 μL of ECF was added, and the solution was vortex-mixed (3−5 s). Five minutes later, 150 μL of dichloromethane and ca. 0.01 g of NaCl were added and the vial was thoroughly shaken for extraction of the derivatives into the organic layer. This phase was transferred into a 200 μL insert adjustable to the liquid sampler vials. About 1.5 μL was injected into the gas chromatographic system.

**GC Analysis.** Separation of L-amino acids was achieved by gas chromatography, carried out with a Chrompack CP 9001 instrument (Chrompack, Middelburg, The Netherlands) equipped with a flame ionization detector (FID) and an automatic liquid sampler (CP-9050, Chrompack). The injector was kept at 250 °C, and the detector was kept at 280 °C. The GC was equipped with an electronic pressure control, allowing programmable gas pressure during the chromatographic run. Helium as carrier gas was used with the following pressure program: increase from initial 50 (1 min hold) to 70 kPa at 4 min. A CP-Sil 19 CB (10 m × 0.25 mm i.d.) WCOT fused-silica capillary column (Varian) was used with the following temperature program: increase from 140 °C (1 min hold) to 280 °C at 40 °C/min (*15*).

The compounds were identified by their retention times and chromatographic comparison with authentic standards. Quantification was based on the internal standard method using L-*p*-chlorophenylalanine.

**Statistics.** For statistical evaluation of total free amino acids and glycine content from quince pulps and peels, a one-way analysis of variance (ANOVA) was used. The mean values evaluated would be considered significantly different at $p < 0.05$.

## RESULTS AND DISCUSSION

All samples (quince fruits and jams) presented a similar qualitative profile with 21 free amino acids identified (**Figure 1**). The retention times obtained for amino acids are shown in **Table 1**.

**Table 4.** Free Amino Acids Composition of Homemade Quince Jams (μg/kg) (Quantification by Internal Standard Technique)[a]

| | quince jams | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | | B | | C | | D | |
| amino acids | mean | SD | mean | SD | mean | SD | mean | SD |
| alanine | 9.5 | 0.33 | 23.1 | 0.79 | 38.6 | 1.98 | 37.6 | 0.81 |
| glycine | 9.4 | 0.16 | 67.9 | 2.13 | 9.6 | 0.38 | 115.0 | 4.52 |
| valine | 1.8 | 0.08 | 4.7 | 0.03 | 25.0 | 0.59 | 10.3 | 0.32 |
| leucine | 0.5 | 0.01 | 1.6 | 0.06 | 4.6 | 0.20 | 4.1 | 0.07 |
| isoleucine | 2.0 | 0.14 | 5.4 | 0.18 | 8.3 | 0.28 | 9.9 | 0.60 |
| proline | 2.2 | 0.09 | 5.2 | 0.15 | 9.9 | 0.20 | 5.4 | 0.25 |
| threonine | 16.2 | 0.32 | 11.6 | 0.48 | 22.6 | 0.90 | 2.3 | 0.12 |
| serine | 4.0 | 0.05 | 12.3 | 0.34 | 23.0 | 0.61 | 16.0 | 0.45 |
| glutamic acid | 6.3 | 0.43 | 23.0 | 0.34 | 49.6 | 1.77 | 5.7 | 0.12 |
| asparagine | 70.2 | 2.92 | 213.3 | 1.21 | 178.8 | 8.75 | 720.5 | 20.85 |
| aspartic acid | 259.0 | 13.02 | 214.9 | 2.70 | 387.9 | 21.55 | 448.4 | 18.58 |
| methionine | 0.2 | 0.01 | 2.2 | 0.09 | 0.1 | 0.01 | 1.0 | 0.01 |
| hydroxyproline | 5.7 | 0.24 | 116.6 | 8.88 | 55.4 | 1.11 | 55.1 | 3.92 |
| phenylalanine | 0.3 | 0.01 | 3.6 | 0.02 | 2.1 | 0.02 | 4.1 | 0.17 |
| cysteine | 25.0 | 0.64 | 7.5 | 0.13 | 55.5 | 1.09 | 6.0 | 0.04 |
| glutamine | 2.3 | 0.09 | 4.5 | 0.05 | 5.5 | 0.06 | 4.1 | 0.10 |
| ornithine | 1.1 | 0.03 | 3.4 | 0.04 | 1.1 | 0.08 | 1.6 | 0.06 |
| lysine | 5.2 | 0.16 | 8.9 | 0.09 | 5.1 | 0.19 | 6.1 | 0.20 |
| histidine | 2.4 | 0.21 | 5.2 | 0.14 | 0.3 | 0.02 | 28.2 | 0.71 |
| tyrosine | 0.3 | 0.01 | 0.2 | 0.01 | 1.4 | 0.01 | 2.3 | 0.17 |
| tryptophan | 0.6 | 0.05 | 1.6 | 0.02 | 0.1 | 0.01 | 1.5 | 0.12 |
| Σ | 424.1 | | 736.8 | | 884.6 | | 1485.2 | |

[a] Values are expressed as the mean of three determinations; SD, standard deviation; Σ, sum of the determined free amino acids.

Since none of the amino acids are volatile enough for direct GC analysis, it is necessary to transform them into volatile derivatives. The chosen amino acids derivatization procedure with ethyl chloroformate is unique in rapidity, although one drawback is the inability to determine arginine (*15*−*19*). Not all reactive groups in the amino acids are altered by action of the reagent. The imino group of arginine remains untouched, which is the reason for the absorption of this amino acid

**Table 5.** Free Amino Acids Composition of Industrially Manufactured Quince Jams (μg/kg) (Quantification by Internal Standard Technique)[a]

| | quince jams | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E | | F | | G | | H | | I | | J | | K | | L | |
| amino acids | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD |
| alanine | 13.3 | 0.47 | 9.2 | 0.29 | 68.1 | 1.05 | 14.4 | 0.37 | 61.2 | 0.65 | 110.3 | 1.75 | 8.3 | 0.16 | 10.1 | 0.61 |
| glycine | 80.2 | 3.38 | 77.5 | 2.10 | 65.3 | 1.92 | 112.7 | 4.39 | 5.5 | 0.06 | 9.6 | 0.20 | 29.1 | 1.53 | 45.2 | 2.57 |
| valine | 4.5 | 0.28 | 2.0 | 0.09 | 19.5 | 0.70 | 3.4 | 0.04 | 15.3 | 0.73 | 3.7 | 0.16 | 7.0 | 0.15 | 6.4 | 0.16 |
| leucine | 1.6 | 0.04 | 1.4 | 0.04 | 5.9 | 0.11 | 1.5 | 0.07 | 2.5 | 0.10 | 1.7 | 0.07 | 1.7 | 0.09 | 3.6 | 0.17 |
| isoleucine | 4.4 | 0.11 | 5.6 | 0.22 | 23.4 | 0.45 | 7.3 | 0.20 | 11.1 | 0.36 | 5.4 | 0.11 | 6.4 | 0.08 | 12.5 | 0.48 |
| proline | 2.6 | 0.06 | 2.9 | 0.10 | 6.2 | 0.12 | 2.4 | 0.09 | 3.5 | 0.14 | 4.6 | 0.18 | 5.3 | 0.22 | 3.1 | 0.02 |
| threonine | 13.1 | 0.33 | 9.9 | 0.33 | 7.7 | 0.23 | 16.4 | 0.50 | 23.2 | 0.57 | 31.0 | 1.07 | 15.0 | 0.77 | 9.9 | 0.39 |
| serine | 7.2 | 0.15 | 10.1 | 0.19 | 16.3 | 0.27 | 5.9 | 0.03 | 14.3 | 0.60 | 15.4 | 0.52 | 13.6 | 0.59 | 10.3 | 0.53 |
| glutamic acid | 4.9 | 0.27 | 26.5 | 0.34 | 25.0 | 1.13 | 29.2 | 0.57 | 76.3 | 3.75 | 61.3 | 1.78 | 38.3 | 1.52 | 16.0 | 0.79 |
| asparagine | 83.7 | 2.53 | 47.2 | 1.22 | 219.0 | 7.71 | 20.3 | 0.60 | 68.1 | 1.57 | 40.7 | 1.42 | 244.7 | 6.83 | 65.9 | 2.31 |
| aspartic acid | 262.1 | 8.06 | 153.7 | 2.81 | 51.0 | 1.13 | 198.9 | 5.59 | 331.1 | 13.07 | 197.9 | 3.65 | 413.9 | 17.09 | 33.7 | 0.94 |
| methionine | 0.1 | 0.01 | 0.1 | 0.01 | 0.4 | 0.02 | 0.4 | 0.01 | 1.5 | 0.01 | 0.4 | 0.02 | 0.1 | 0.01 | 0.3 | 0.01 |
| hydroxyproline | 5.2 | 0.11 | 11.9 | 0.80 | 41.5 | 0.65 | 11.5 | 0.19 | 325.5 | 11.71 | 11.9 | 0.16 | 5.8 | 0.30 | 27.9 | 0.74 |
| phenylalanine | 1.5 | 0.09 | 1.0 | 0.06 | 4.0 | 0.12 | 2.2 | 0.06 | 2.2 | 0.14 | 2.6 | 0.03 | 6.7 | 0.19 | 6.7 | 0.05 |
| cysteine | 9.3 | 0.60 | 8.1 | 0.27 | 0.4 | 0.01 | 9.6 | 0.22 | 119.1 | 4.14 | 38.9 | 1.65 | 21.8 | 0.77 | 7.6 | 0.68 |
| glutamine | 5.0 | 0.20 | 6.4 | 0.17 | 5.8 | 0.23 | 7.6 | 0.54 | 17.2 | 1.17 | 8.6 | 0.36 | 7.1 | 0.04 | 4.8 | 0.32 |
| ornithine | 0.7 | 0.01 | 1.0 | 0.05 | 4.8 | 0.30 | 0.6 | 0.02 | 0.6 | 0.03 | 1.3 | 0.03 | 0.5 | 0.01 | 3.7 | 0.33 |
| lysine | 6.5 | 0.26 | 7.5 | 0.16 | 14.9 | 0.35 | 3.7 | 0.07 | 3.5 | 0.15 | 8.2 | 0.16 | 8.3 | 0.25 | 8.9 | 0.38 |
| histidine | 5.6 | 0.43 | 7.7 | 0.04 | 10.7 | 0.61 | 1.4 | 0.06 | 1.5 | 0.02 | 4.0 | 0.09 | 5.8 | 0.06 | 27.0 | 0.61 |
| tyrosine | 0.6 | 0.01 | 1.0 | 0.06 | 1.9 | 0.10 | 0.3 | 0.01 | 3.1 | 0.14 | 0.7 | 0.01 | 0.5 | 0.01 | 1.1 | 0.06 |
| tryptophan | 0.6 | 0.01 | 0.1 | 0.01 | 1.3 | 0.05 | 1.6 | 0.07 | 0.2 | 0.01 | 0.2 | 0.01 | 2.5 | 0.04 | 4.6 | 0.24 |
| Σ | 512.6 | | 390.6 | | 593.0 | | 451.0 | | 1086.5 | | 558.5 | | 842.7 | | 309.2 | |

| | quince jams | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | | N | | O | | P | | Q | | R | | S | | T | |
| amino acids | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD |
| alanine | 7.4 | 0.30 | 21.8 | 0.87 | 10.7 | 0.24 | 15.9 | 1.01 | 198.6 | 6.04 | 10.5 | 0.06 | 24.1 | 0.33 | 68.4 | 0.62 |
| glycine | 21.9 | 0.99 | 58.4 | 2.11 | 86.9 | 2.21 | 9.2 | 0.44 | 86.1 | 1.79 | 102.0 | 4.12 | 57.2 | 1.46 | 9.8 | 0.49 |
| valine | 5.4 | 0.12 | 6.5 | 0.33 | 7.5 | 0.20 | 7.2 | 0.29 | 6.3 | 0.24 | 7.3 | 0.21 | 5.0 | 0.02 | 6.9 | 0.23 |
| leucine | 1.8 | 0.06 | 1.5 | 0.06 | 2.5 | 0.07 | 3.4 | 0.10 | 0.8 | 0.04 | 1.6 | 0.04 | 1.4 | 0.01 | 5.1 | 0.06 |
| isoleucine | 5.2 | 0.13 | 6.2 | 0.21 | 9.4 | 0.18 | 7.7 | 0.17 | 80.1 | 0.42 | 7.9 | 0.19 | 5.9 | 0.27 | 9.3 | 0.04 |
| proline | 2.5 | 0.09 | 2.2 | 0.05 | 3.8 | 0.05 | 4.0 | 0.08 | 1.4 | 0.08 | 2.2 | 0.10 | 1.9 | 0.11 | 10.2 | 0.15 |
| threonine | 19.1 | 0.39 | 18.0 | 0.34 | 20.2 | 0.35 | 29.1 | 1.29 | 8.2 | 0.08 | 21.1 | 1.04 | 55.5 | 2.12 | 44.5 | 0.97 |
| serine | 7.0 | 0.09 | 11.9 | 0.24 | 10.8 | 0.73 | 36.2 | 1.56 | 8.9 | 0.48 | 6.3 | 0.17 | 5.1 | 0.29 | 29.3 | 1.21 |
| glutamic acid | 9.6 | 0.11 | 20.8 | 0.99 | 24.0 | 0.86 | 18.5 | 0.92 | 23.4 | 0.16 | 19.4 | 0.39 | 10.3 | 0.15 | 46.2 | 1.82 |
| asparagine | 82.4 | 3.26 | 142.5 | 1.07 | 174.1 | 1.47 | 95.0 | 3.52 | 125.3 | 4.88 | 80.8 | 0.61 | 92.3 | 5.30 | 293.7 | 10.16 |
| aspartic acid | 215.3 | 3.11 | 324.8 | 6.25 | 390.0 | 3.55 | 158.9 | 11.20 | 151.7 | 0.19 | 291.5 | 4.76 | 392.3 | 16.07 | 391.1 | 13.17 |
| methionine | 0.1 | 0.01 | 0.9 | 0.06 | 0.2 | 0.01 | 0.1 | 0.01 | 0.1 | 0.01 | 0.1 | 0.01 | 0.2 | 0.02 | 0.3 | 0.01 |
| hydroxyproline | 17.9 | 0.30 | 96.3 | 3.29 | 166.0 | 5.55 | 68.7 | 3.20 | 21.8 | 0.24 | 5.1 | 0.33 | 144.5 | 7.34 | 26.8 | 0.79 |
| phenylalanine | 2.5 | 0.12 | 3.4 | 0.10 | 2.1 | 0.02 | 1.0 | 0.03 | 1.3 | 0.03 | 1.7 | 0.02 | 0.3 | 0.02 | 4.2 | 0.10 |
| cysteine | 19.4 | 0.22 | 25.6 | 0.97 | 20.2 | 1.21 | 68.1 | 3.24 | 15.4 | 0.13 | 14.9 | 0.66 | 9.5 | 0.25 | 41.0 | 1.11 |
| glutamine | 5.6 | 0.06 | 0.3 | 0.02 | 16.0 | 0.94 | 16.0 | 0.60 | 13.2 | 0.55 | 10.9 | 0.72 | 1.0 | 0.02 | 21.3 | 0.26 |
| ornithine | 1.0 | 0.01 | 1.5 | 0.05 | 1.4 | 0.08 | 1.2 | 0.02 | 0.6 | 0.02 | 1.4 | 0.05 | 0.5 | 0.04 | 1.2 | 0.03 |
| lysine | 11.0 | 0.18 | 12.7 | 0.34 | 9.4 | 0.41 | 4.8 | 0.01 | 2.9 | 0.07 | 7.9 | 0.04 | 2.0 | 0.03 | 8.2 | 0.12 |
| histidine | 2.9 | 0.18 | 7.9 | 0.01 | 8.4 | 0.08 | 0.9 | 0.06 | 1.9 | 0.01 | 10.7 | 0.47 | 4.4 | 0.25 | 7.8 | 0.04 |
| tyrosine | 0.3 | 0.02 | 1.0 | 0.06 | 1.7 | 0.10 | 2.3 | 0.07 | 0.3 | 0.02 | 0.4 | 0.02 | 0.7 | 0.03 | 3.0 | 0.10 |
| tryptophan | 1.1 | 0.03 | 0.1 | 0.01 | 1.3 | 0.04 | 0.1 | 0.01 | 0.8 | 0.01 | 1.7 | 0.01 | 0.3 | 0.01 | 1.0 | 0.03 |
| Σ | 439.1 | | 764.2 | | 966.7 | | 548.1 | | 749.1 | | 605.7 | | 814.5 | | 1029.3 | |

[a] Values are expressed as the mean of three determinations; SD, standard deviation; Σ, sum of the determined free amino acids.

derivative in the column (*15*−*19*). For the determination of arginine, an additional reaction step would be necessary or, alternatively, its conversion into ornithine by arginase before derivatization (*15*) would be required.

Because the free amino acid pattern is typical of a fruit and hence can be utilized for the analytical characterization of a fruit product (*1*), samples of quince fruit (pulp and peel) from seven different geographic origins from Portugal were analyzed. As expected, quince fruit is a food matrix with very low free amino acids content. The sum of the 21 free amino acids ranged from approximately 316 to 1357 μg/kg for quince pulps and from 512 to 1820 μg/kg for quince peels (**Tables 2** and **3**). As a general rule, the amount of free amino acids was higher in peels than in the corresponding pulps, with the exceptions of quinces from Vila Real and Pinhel. Nevertheless, the difference between the mean value of total free amino acids content from quince pulps (807.2 μg/kg; $n = 7$) and the mean value from quince peels (1009.9 μg/kg; $n = 7$) was not significant ($p > 0.05$). All peels, except the one from Amarante, were characterized by higher absolute and percent amounts of glycine than the correspondent pulps, although the difference between the mean value of the glycine content (and percent) from quince pulps (69.8 μg/kg; $n = 7$) and from quince peels (217.6 μg/kg; $n = 7$) was not significant ($p > 0.05$).

In what concerns the quince pulps, generally the three most abundant free amino acids were aspartic acid, hydroxyproline, and asparagine. Free amino acids such as methionine, ornithine, and tyrosine were always present in very low amounts (< 1%).

As a general rule, the three most abundant compounds present in quince peels were glycine, aspartic acid, and asparagine. The

content in leucine, methionine, ornithine, and tyrosine was always very low (< 1%).

It seems that the free amino acids content was related to the quince fruit geographical origin. However, other factors, such as cultural practices and degree of maturation, may have caused the observed differences.

Because pear and apple also belong to the *Rosaceae* family, it is important to compare the results herein obtained with those published for pear and apple. Asparagine, aspartic acid, glutamic acid, and serine are the major free amino acids of apple (*1, 3, 4*) and account for roughly 80% of the total free amino acids content (*3, 4*). According to Belitz and Grosch (*1*), the main free amino acid of pear is proline, but considerable percentages of aspartic acid, asparagine, glutamic acid, serine, and alanine are also present. Gomis et al. (*3*) reported that secondary amino acids, such as proline, are present in small amounts in apple, which means that pear is distinctive for its relatively high levels of proline compared to apple (*20*). According to Lea (*20*) and van Gorsel et al. (*21*), asparagine and glutamic acid account for the majority of free amino acids in pear and apple juices, followed by aspartic acid (*20, 21*). Pear juice also contains considerable amounts of serine, proline, valine, isoleucine, cysteine, and histidine (*21*). In what concerns apple juice, it also presents considerable content in serine, alanine, isoleucine, and histidine (*20, 21*). van Gorsel et al. (*21*) did not found any traces of tyrosine in apple juice or methionine in pear juice.

It seems that quince has some similarities with apple and pear; asparagine and aspartic acid are usually two of the major free amino acids. Sometimes, as in apple and/or pear, glutamic acid, cysteine, and histidine are the most abundant free amino acids. A high content of glycine and/or a comparatively high amount of hydroxyproline seems to be characteristic of quince fruit. Proline is present in small levels in quince (≤2.4%), such as in apple.

A total of 20 quince jam samples (4 homemade and 16 industrially manufactured) were analyzed. For all homemade quince jams, the two most abundant free amino acids were aspartic acid and asparagine and the third was cysteine, hydroxyproline, or glycine (**Table 4**). Many free amino acids (leucine, isoleucine, methionine, phenylalanine, glutamine, ornithine, tyrosine, and tryptophan) were present in very low amounts (<1%).

As in quince pulps and peels, in the industrially manufactured quince jam samples, the three major free amino acids were generally aspartic acid, asparagine, and glycine or hydroxyproline (**Table 5**). According to Portuguese legislation (*22*), quince jam is the food product of the homogeneous and consistent mixture, obtained exclusively by boiling quince mesocarp with sugars. It is possible that samples that had glycine as one of the three major free amino acids (samples D–H, L, M, and R) were prepared with unpeeled quinces. In some cases, amino acids such as alanine, glutamic acid, and cysteine were also among the three major amino acids. Probably the high alanine percentages (>8%) (samples G, J, and Q) were false because of addition of pear. A high content of proline could also be related to pear addition, but all quince jams had low percentages of this free amino acid (≤1%). Proline, methionine, and tyrosine content was always inferior to 1%.

In **Table 6** are presented the mean values (and SD) of free amino acids obtained for pulps, peels, and jams. Considering the total free amino acid content of quince pulps and peels and the usual fruit content of quince jams (ranging from 40% to 50%), some samples (D, I, O, and T) had higher amounts than expected. This may be due to the natural variability of fruits and/or to a possible hydrolysis of proteins, peptides, or other compounds with amino acids in their constitution, which can occur during the thermal processing (in acid medium).

**Table 6.** Free Amino Acids Composition of Quince Pulps, Peels, and Jams (μg/kg) (Mean Values)[a]

| | samples | | | | | |
|---|---|---|---|---|---|---|
| | pulps | | peels | | jams | |
| amino acids | mean | SD | mean | SD | mean | SD |
| alanine | 32.7 | 23.58 | 31.3 | 19.59 | 38.1 | 46.71 |
| glycine | 69.8 | 66.98 | 217.6 | 133.97 | 52.9 | 37.78 |
| valine | 17.9 | 14.58 | 23.3 | 18.91 | 7.8 | 5.82 |
| leucine | 5.6 | 4.84 | 5.3 | 5.99 | 2.4 | 1.50 |
| isoleucine | 21.4 | 21.97 | 42.5 | 38.94 | 11.7 | 16.69 |
| proline | 9.4 | 6.07 | 11.0 | 7.29 | 4.1 | 2.44 |
| threonine | 13.1 | 11.41 | 15.5 | 16.36 | 19.7 | 12.70 |
| serine | 35.1 | 23.96 | 44.0 | 45.42 | 13.2 | 8.20 |
| glutamic acid | 64.2 | 50.11 | 84.3 | 73.48 | 26.7 | 19.06 |
| asparagine | 133.4 | 91.35 | 121.6 | 57.99 | 152.9 | 153.10 |
| aspartic acid | 189.9 | 133.12 | 198.3 | 115.08 | 263.4 | 120.72 |
| methionine | 2.4 | 1.72 | 1.8 | 1.29 | 0.4 | 0.56 |
| hydroxyproline | 102.1 | 95.12 | 87.3 | 73.84 | 61.1 | 78.83 |
| phenylalanine | 6.5 | 5.56 | 5.7 | 4.96 | 2.7 | 1.81 |
| cysteine | 33.0 | 22.21 | 31.5 | 13.48 | 26.1 | 28.01 |
| glutamine | 14.9 | 13.16 | 12.4 | 10.68 | 8.2 | 5.77 |
| ornithine | 2.1 | 2.69 | 2.7 | 2.86 | 1.5 | 1.16 |
| lysine | 15.6 | 10.94 | 19.2 | 15.55 | 7.3 | 3.29 |
| histidine | 27.6 | 39.48 | 41.2 | 29.67 | 7.2 | 7.64 |
| tyrosine | 1.0 | 1.12 | 1.6 | 1.94 | 1.2 | 0.93 |
| tryptophan | 9.4 | 7.89 | 11.8 | 18.86 | 1.1 | 1.08 |
| Σ | 807.2 | 363.59 | 1009.9 | 490.59 | 709.6 | 287.67 |

[a] SD, standard deviation; Σ, sum of the determined free amino acids.

In conclusion, although the free amino acid profile is not as suitable as the phenolic one (*9, 13*) for the detection of false readings of quince jam with quince peel and/or pear and apple, it may be a useful tool for this purpose.

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# COMPOSITION OF QUINCE (*CYDONIA OBLONGA* MILLER) SEEDS: PHENOLICS, ORGANIC ACIDS AND FREE AMINO ACIDS

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Phenolic compounds, organic acids and free amino acids of quince seeds were determined by HPLC/DAD, HPLC/UV and GC/FID, respectively. Quince seeds presented a phenolic profile composed of 3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic and 3,5-dicaffeoylquinic acids, lucenin-2, vicenin-2, stellarin-2, isoschaftoside, schaftoside, 6-*C*-pentosyl-8-*C*-glucosyl chrysoeriol and 6-*C*-glucosyl-8-*C*-pentosyl chrysoeriol. Six identified organic acids constituted the organic acid profile of quince seeds: citric, ascorbic, malic, quinic, shikimic and fumaric acids. The free amino acid profile was composed of 21 identified free amino acids and the three most abundant were glutamic and aspartic acids and asparagine.

*Keywords:* *Cydonia oblonga* Miller; Quince seeds; Phenolics; Organic acids; Free amino acids

## INTRODUCTION

Quince is the fruit of a deciduous tree of the Rosaceae family, *Cydonia oblonga* Miller. Due to its hardness, acidity and astringency, it is not edible fresh; nevertheless, it is often used to prepare jam.

Some chemical studies have been previously carried out for quince fruit. In 1979 and 1986, the volatile constituents of quince were analysed by GC/MS [1,2]. Through analysis of the phenolic profile, glucosides of procyanidin polymers have been identified in this fruit [3,4] and, recently, it has been possible to discriminate quince pulp and peel by the analysis of phenolics [5]. In 2002, an HPLC/UV method was developed for the determination of organic acids in quince fruit (pulp and peel) [6]. More recently,

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a GC/FID methodology was developed for the analysis of free amino acids in the same matrices [7].

As far as we know, for quince seeds, few chemical studies have been developed. Ion-trap HPLC-PAD-ESI/MS/MS has been used in a qualitative study of *C*-glycosyl flavones in these seeds [8]. As part of a continuing study of quince, the work herein represents a contribution to the composition of quince seeds, in terms of phenolic compounds, organic acids and free amino acids.

## RESULTS AND DISCUSSION

### Phenolic Compounds

Quince seeds presented a phenolic profile composed of 3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic and 3,5-dicaffeoylquinic acids, lucenin-2 (6,8-di-*C*-glucosyl luteolin), vicenin-2 (6,8-di-*C*-glucosyl apigenin), stellarin-2 (6,8-di-*C*-glucosyl chrysoeriol), isoschaftoside (6-*C*-arabinosyl-8-*C*-glucosyl apigenin), schaftoside (6-*C*-glucosyl-8-*C*-arabinosyl apigenin), 6-*C*-pentosyl-8-*C*-glucosyl chrysoeriol and 6-*C*-glucosyl-8-*C*-pentosyl chrysoeriol (Fig. 1).

While quince pulps contained mainly caffeoylquinic acids and quince peels presented both caffeoylquinic acids and several flavonol glycosides in great amounts [5], quince seeds were characterised by the presence of caffeoylquinic acids and, specially, *C*-glycosyl flavones. Caffeoylquinic acids ranged from *ca.* 34 to 37% of the determined phenolics, with 5-*O*-caffeoylquinic acid being the most abundant (from 19 to 24%), while *C*-glycosyl flavones ranged from 63 to 66%, with isoschaftoside being the major one (*ca.* 18%). The total phenolic content was *ca.* 0.1 g/kg (Table I), which is

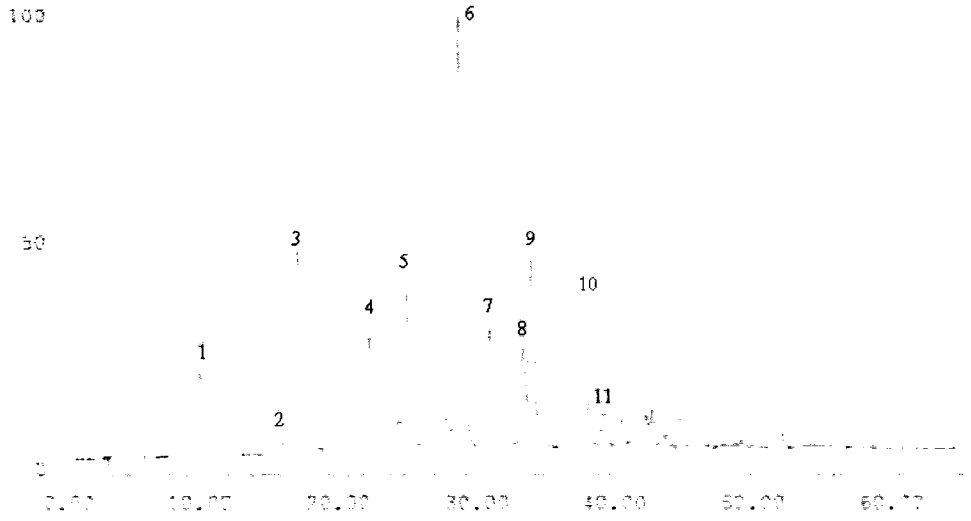


FIGURE 1 HPLC phenolic profile of quince seeds (350 nm). 1 – 3-*O*-caffeoylquinic acid; 2 – 4-*O*-caffeoylquinic acid; 3 – 5-*O*-caffeoylquinic acid; 4 – lucenin-2; 5 – vicenin-2; 6 – stellarin-2; 7 – isoschaftoside; 8 – schaftoside; 9 – 6-*C*-pentosyl-8-*C*-glucosyl chrysoeriol; 10 – 6-*C*-glucosyl-8-*C*-pentosyl chrysoeriol; 11 – 3,5-dicaffeoylquinic acid.

TABLE I Phenolic composition of quince seeds (mg/kg)

| *Phenolic compounds* | *Samples* | | | | | |
|---|---|---|---|---|---|---|
| | *2000* | | *2001* | | *2002* | |
| | *Mean* | *SD* | *Mean* | *SD* | *Mean* | *SD* |
| 3-*O*-caffeoylquinic acid | 6.1 | 0.06 | 10.1 | 0.07 | 12.4 | 0.40 |
| 4-*O*-caffeoylquinic acid | 1.4 | 0.08 | 1.1 | 0.03 | 1.7 | 0.01 |
| 5-*O*-caffeoylquinic acid | 27.8 | 0.24 | 20.0 | 0.24 | 24.6 | 0.69 |
| Lucenin-2 | 6.7 | 0.07 | 7.6 | 0.13 | 5.2 | 0.23 |
| Vicenin-2 | 13.9 | 0.17 | 12.5 | 0.10 | 14.2 | 0.14 |
| Stellarin-2 | 20.6 | 0.21 | 18.9 | 0.42 | 20.5 | 0.37 |
| Isoschaftoside | 10.8 | 0.03 | 8.7 | 0.13 | 10.0 | 0.07 |
| Schaftoside | nq | | 6.4 | 0.01 | 7.1 | 0.11 |
| 6-*C*-pentosyl-8-*C*-glucosyl chrysoeriol | 15.4 | 0.13 | 9.0 | 0.14 | 8.0 | 0.38 |
| 6-*C*-glucosyl-8-*C*-pentosyl chrysoeriol | 8.2 | 0.26 | 7.7 | 0.14 | 7.7 | 0.43 |
| 3,5-*O*-dicaffeoylquinic acid | 5.6 | 0.01 | 5.3 | 0.11 | 4.4 | 0.10 |
| $\sum$ | 116.4 | | 107.4 | | 115.8 | |

Values are expressed as mean of the three determinations; *SD* – standard deviation; $\sum$ – sum of the determined phenolic compounds; nq – not quantified.

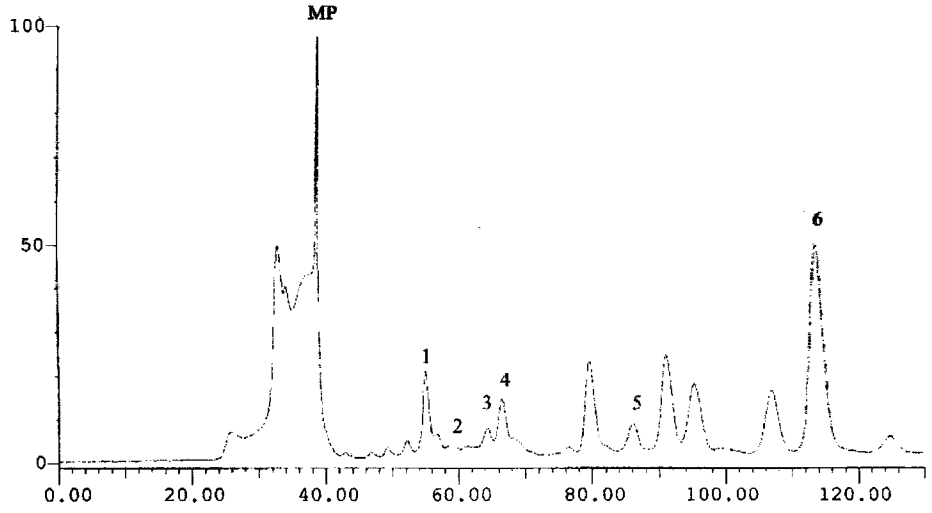


FIGURE 2 HPLC organic acid profile of quince seeds. MP – mobile phase; 1 – citric acid; 2 – ascorbic acid; 3 – malic acid; 4 – quinic acid; 5 – shikimic acid; 6 – fumaric acid.

similar to that obtained for pulps, but much lower than the one that was present in the peels [5].

## Organic Acids

Seeds presented an organic acid profile composed of six identified organic acids: citric, ascorbic, malic, quinic, shikimic and fumaric acids (Fig. 2). Contrary to both quince pulp and peel [6], oxalic acid was not detected in quince seeds.

TABLE II Organic acid composition of quince seeds (mg/kg)

| *Organic acids* | *Samples* | | | | | |
|---|---|---|---|---|---|---|
| | *2000* | | *2001* | | *2002* | |
| | *Mean* | *SD* | *Mean* | *SD* | *Mean* | *SD* |
| Citric acid | 213.0 | 10.11 | 164.9 | 4.08 | 93.2 | 1.15 |
| Ascorbic acid | 209.1 | 2.19 | 83.9 | 0.72 | 86.1 | 8.16 |
| Malic + quinic acids | 352.0 | 0.40 | 383.0 | 24.34 | 281.8 | 14.36 |
| Shikimic acid | 1.6 | 0.02 | 1.9 | 0.05 | 1.4 | 0.17 |
| Fumaric acid | 6.2 | 0.26 | 3.3 | 0.05 | 1.8 | 0.04 |
| $\sum$ | 781.9 | | 636.9 | | 464.2 | |

Values are expressed as mean of the three determinations; *SD* – standard deviation; $\sum$ – sum of the determined organic acids.

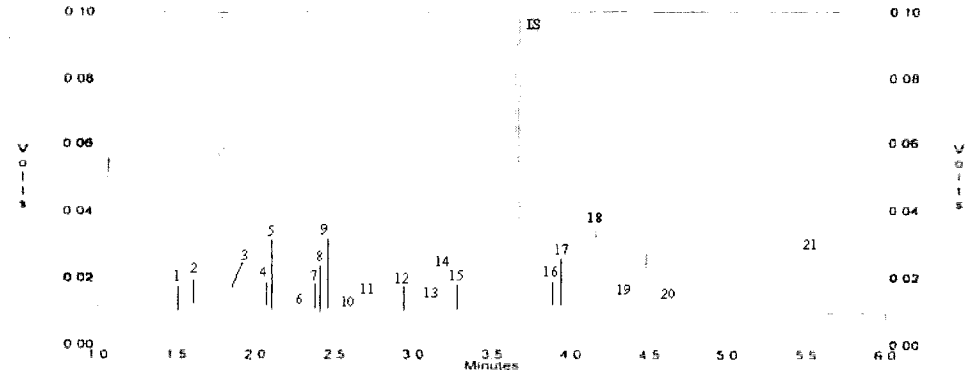


FIGURE 3 GC free amino acid profile of quince seeds. 1 – alanine; 2 – glycine; 3 – valine; 4 – leucine; 5 – isoleucine; 6 – proline; 7 – threonine; 8 – serine; 9 – glutamic acid; 10 – asparagine; 11 – aspartic acid; 12 – methionine; 13 – hydroxyproline; 14 – phenylalanine; 15 – cysteine; IS – internal standard (L-*p*-chlorophenylalanine); 16 – glutamine; 17 – ornithine; 18 – lysine; 19 – histidine; 20 – tyrosine; 21 – tryptophan.

The total organic acid content ranged from *ca.* 0.5 to 0.8 g/kg (Table II), which is much lower than the one that was obtained for quince pulp and peel collected in 2000 [6]. The sum of malic acid plus quinic acid represented 45 to 61% of the total organic acid content, while in quince pulp and peel, malic and quinic acids always constituted more than 93% [6]. Citric and ascorbic acids in seeds were also present in great percentages, the first acid ranged from 20 to 27% and the second one ranged from 13 to 27% of the determined organic acids, while in pulp and peel, these acids were present in smaller percentages (< 6%) [6]. In seeds, as previously reported for quince pulp and peel [6], shikimic and fumaric acids were present in very low amounts (< 1%).

## Free Amino Acids

Samples presented a free amino acid profile composed of 21 identified free amino acids. It was not possible to quantify valine, once it coeluted with an interference compound (Fig. 3).

The sum of the 21 free amino acids ranged from *ca.* 1.3 to 1.7 mg/kg (Table III). The total free amino acids content was higher than that found in pulp and peel from fruits collected in 2000 (mean values of 0.8 and 1.0 mg/kg, respectively). As it happened with

TABLE III Free amino acids composition of quince seeds (μg/kg)

| *Amino acids* | *Samples* | | | | | |
|---|---|---|---|---|---|---|
| | *2000* | | *2001* | | *2002* | |
| | *Mean* | *SD* | *Mean* | *SD* | *Mean* | *SD* |
| Alanine | 23.5 | 0.89 | 20.1 | 0.88 | 72.7 | 6.31 |
| Glycine | 34.6 | 1.31 | 42.5 | 1.48 | 15.8 | 0.16 |
| Valine | nq | – | nq | – | nq | – |
| Leucine | 5.4 | 0.13 | 15.0 | 0.60 | 16.4 | 0.41 |
| Isoleucine | 3.6 | 0.07 | 9.1 | 0.21 | 7.1 | 0.04 |
| Proline | 6.5 | 0.24 | 8.7 | 0.21 | 8.0 | 0.23 |
| Threonine | 25.4 | 1.11 | 115.0 | 3.24 | 56.7 | 0.37 |
| Serine | 71.3 | 1.54 | 29.7 | 1.15 | 42.8 | 3.40 |
| Glutamic acid | 314.2 | 8.92 | 525.3 | 26.51 | 740.5 | 31.84 |
| Asparagine | 106.8 | 4.04 | 230.2 | 4.84 | 191.3 | 6.39 |
| Aspartic acid | 470.1 | 8.35 | 201.7 | 5.48 | 367.4 | 11.92 |
| Methionine | 2.2 | 0.07 | 5.1 | 0.05 | 3.2 | 0.13 |
| Hydroxyproline | 106.5 | 4.96 | 161.8 | 1.70 | 12.3 | 0.77 |
| Phenylalanine | 11.1 | 0.28 | 22.9 | 0.56 | 21.5 | 0.04 |
| Cysteine | 4.9 | 0.09 | 2.9 | 0.01 | 2.7 | 0.06 |
| Glutamine | 9.5 | 0.43 | 21.2 | 0.26 | 60.0 | 2.29 |
| Ornithine | 2.3 | 0.06 | 6.3 | 0.22 | 1.0 | 0.02 |
| Lysine | 45.6 | 1.96 | 60.5 | 1.80 | 21.7 | 0.40 |
| Histidine | 28.8 | 0.99 | 50.6 | 1.43 | 22.9 | 0.02 |
| Tyrosine | 4.6 | 0.05 | 7.8 | 0.34 | 7.7 | 0.61 |
| Tryptophan | 68.6 | 4.86 | 47.4 | 1.25 | 49.5 | 3.90 |
| $\sum$ | 1345.3 | | 1583.6 | | 1721.1 | |

Values are expressed as mean of the three determinations; *SD* standard deviation; $\sum$ sum of the determined free amino acids; nq not quantified.

some pulp and peel (data not shown), the three major free amino acids were glutamic acid, aspartic acid and asparagine, which constituted 60 to 75% of the totality of free amino acids.

In conclusion, the phenolic profile constitutes the most useful chemical parameter for the discrimination of quince seed, pulp and peel. Seeds can be characterized by the presence of several *C*-glycosyl flavones (which are absent in pulp and peel). In what concerns to organic acid composition, in seeds, malic and quinic acids are not so predominant as in pulp and peel (citric and ascorbic acids are also present in considerable amounts). The sum of all acids is, nevertheless, much lower. The free amino acid profile does not seem to be so characteristic of this part of quince fruit.

## EXPERIMENTAL

### Samples

Healthy quince fruits were collected in several different locations of Northern (Amarante, Baião, Vila Real and Bragança) and Central Portugal (Viseu, Pinhel and Covilhã), in the years of 2000, 2001 and 2002. Seeds were freeze-dried using a Labconco 4.5 apparatus (Kansas City, MO). Owing to plant material scarcity, equal portions of lyophilised seeds from each geographical region were combined and pulverized.

### Standards

The standards were from Sigma (St. Louis, MO, USA) and from Extrasynthése (Genay, France). Methanol, formic and hydrochloric acids were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). Ethyl chloroformate (ECF) was from Aldrich (Steinheim, Germany) and pyridine from Fluka (Neu-Ulm, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

### Solid-phase Extraction (SPE) Columns

The ISOLUTE C18 non end-capped (NEC) SPE columns (50 μm particle size, 60 Å porosity; 10 g sorbent mass/70 mL reservoir volume) were purchased from International Sorbent Technology Ltd (Mid Glamorgan, UK). The benzenesulfonic SCX Spe-ed SPE cartridges (200 mg; 3 mL) were obtained from Applied Separations (Allentown, USA).

### Extraction of Phenolic Compounds

The extraction of phenolics was achieved as previously reported [5] and included a C18 NEC SPE cleaning step.

### Extraction of Organic Acids

The sample preparation was simple, involving only extraction with methanol (40°C) and filtration through a C18 NEC SPE cartridge, as reported by Silva *et al.* [6].

### Extraction of Free Amino Acids

According to Silva *et al.* [7], the extraction of L-amino acids was simple, including a SCX SPE purification step.

### Derivatization Procedure

The derivatization of L-amino acids was carried out as reported previously [7].

### HPLC Analysis of Phenolics

The extracts were analysed on an analytical HPLC unit (Gilson), using an Spherisorb ODS2 (25.0 × 0.46 cm; 5 μm, particle size) column [5]. Detection was achieved with a Gilson DAD.

Phenolics were identified by comparison of their retention times, UV-Vis spectra in the 200 400 nm range, and MS with those obtained from standards [5,8].

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3- and 4-*O*-caffeoylquinic, and 3,5-dicaffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid. Luteolin, apigenin and chrysoeriol derivatives were quantified as 7-*O*-glucosyl luteolin, 7-*O*-glucosyl apigenin and chrysoeriol, respectively.

### HPLC Analysis of Organic Acids

The separation was carried out as previously reported [6] with an analytical HPLC unit (Gilson), using an ion exclusion column Nucleogel® Ion 300 OA (300 × 7.7 mm). 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. Malic and quinic acids were quantified together and as malic acid. The other acids were quantified as themselves.

### GC Analysis of Free Amino Acids

The extracts were analysed on a Chrompack CP 9001 instrument (Chrompack, Middelburg, The Netherlands) equipped with a flame ionisation detector (FID), and an automatic liquid sampler (CP-9050, Chrompack) [7].

The amino acids were identified by their retention times and chromatographic comparison with authentic standards. Quantification was based on the internal standard method using L-*p*-chlorophenylalanine.

### *Acknowledgements*

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# VI. INFLUÊNCIA DO PROCESSAMENTO TÉRMICO NOS PERFIS EM COMPOSTOS FENÓLICOS, ÁCIDOS ORGÂNICOS E AMINOÁCIDOS LIVRES

## Índice

Publicação n.º 9 – "Influence of jam processing upon the contents of phenolics, organic acids and free amino acids in quince fruit (*Cydonia oblonga* Miller)"
*Eur. Food Res. Technol.*, 2004, 218, 385-389 139

Eur Food Res Technol (2004) 218:385–389
DOI 10.1007/s00217-003-0845-6

ORIGINAL PAPER

**Branca Maria Silva · Paula Branquinho Andrade · Ana Claúdia Gonçalves · Rosa Maria Seabra · Maria Beatriz Oliveira · Margarida Alice Ferreira**

# Influence of jam processing upon the contents of phenolics, organic acids and free amino acids in quince fruit (*Cydonia oblonga* Miller)

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**Abstract** Phenolic compounds, organic acids and free amino acids of quince were evaluated, before and after jam processing, to test the effect of thermal processing in these compounds. In addition, the composition of jams prepared with peeled and unpeeled quinces was compared. Phenolics, organic acids and free amino acids were analysed by HPLC/DAD, HPLC/UV and GC/FID, respectively.

**Keywords** *Cydonia oblonga* Miller · Quince fruit · Jam · Phenolic compounds · Organic acids · Free amino acids

## Introduction

In recent years it has become evident that significant health risks and benefits are associated with dietary food choice [1]. Nutritional studies recommend the regular consumption of fruits and vegetables, which constitute an essential part of the Mediterranean diet, to favour a healthy quality of life [2]. Among fruits, quince is an important source of health-promoting constituents, such as phenolics, organic acids and amino acids. Although fresh quince fruit is not edible when raw, because of its hardness, bitterness and astringency, it is very appreciated in Portugal for its jam. According to the Portuguese legislation [3], quince jam is the food product of the homogeneous and consistent mixture, obtained exclusively by boiling quince mesocarp with sugars.

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Some chemical studies have been developed on quince fruit and its derivatives. The usefulness of phenolic compounds in the determination of genuineness of quince puree [4], jam [5, 6]and jelly [7] has been reported. Glucosides of procyanidin polymers have been previously identified in this fruit [8, 9]. Recently, it has become possible to discriminate quince pulp and peel by the analysis of its phenolic compounds [10]. In 2002, an HPLC/UV method was developed for the determination of organic acids in quince fruit and jam [11]. More recently, a GC/FID method was developed for the determination of free amino acids in the same matrices [12].

The main purpose of this study was to investigate the influence of jam processing in quince composition, in terms of phenolic compounds, organic acids and free amino acids. As a sequence of previous studies on the distinction between quince pulp and peel [10, 11], we tested the possibility of detecting peel in quince jams by using the referred compounds. With this aim, two quince jams were also prepared and analysed, one of them from peeled quinces and another from unpeeled fruit.

## Materials and methods

### Samples

Healthy quince fruit were collected in Amarante (northern Portugal). Some fruit were separated into pulp and peel and each part of the fruit was cut into thin slices and freeze-dried. Lyophilisation was carried out using a Labconco 4.5 apparatus (Kansas City, MO). Other fruit were used to prepare quince jams.

A quince jam (jam A) was prepared in the laboratory by boiling fresh quince pulp with sugar (in the proportion of 50:50), for approximately 90 min. Another quince jam (jam B) was similarly prepared, but using unpeeled quinces.

### Standards

The standards were from Sigma (St. Louis, MO, USA) and from Extrasynthése (Genay, France). Methanol, formic and hydrochloric

acids were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). Ethyl chloroformate (ECF) was from Aldrich (Steinheim, Germany) and pyridine from Fluka (Neu-Ulm, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

### Solid-phase extraction (SPE) columns

The ISOLUTE C18 non end-capped (NEC) SPE columns (50 μm particle size, 60 Å porosity; 10 g sorbent mass/70 mL reservoir volume) were purchased from International Sorbent Technology (Mid Glamorgan, UK). The benzenesulfonic SCX Spe-ed SPE cartridges (200 mg; 3 mL) were obtained from Applied Separations (Allentown, USA).

### Extraction of phenolic compounds

The extraction of phenolics was achieved as previously reported [6, 10] and included a C18 NEC SPE cleaning step.

### Extraction of organic acids

The sample preparation was simple, involving only extraction with methanol (40 °C) and filtration through a C18 NEC SPE cartridge, as reported by Silva et al. [11].

### Extraction of free amino acids

According to Silva et al. [12], the extraction of L-amino acids was simple, including a SCX SPE purification step.

### Derivatisation procedure

The derivatisation of L-amino acids was carried out as reported previously [12].

### HPLC analysis of phenolics

The extracts were analysed on an analytical HPLC unit (Gilson), using a Spherisorb ODS2 (25.0x0.46 cm; 5 μm, particle size) column [4, 5, 7, 10]. Detection was achieved with a Gilson DAD.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3- and 4-*O*-caffeoylquinic, and 3,5-dicaffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid. Kaempferol glycoside and kaempferol glycosides acylated with *p*-coumaric acid were quantified as kaempferol 3-glucoside. Quercetin glycosides acylated with *p*-coumaric acid were quantified as quercetin 3-galactoside. The other compounds were quantified as themselves.

### HPLC analysis of organic acids

The separation was carried out as previously reported [11] with an analytical HPLC unit (Gilson), using an ion exclusion column Nucleogel Ion 300 OA (300x7.7 mm) column. 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. Malic and quinic acids were quantified together and as malic acid. The other acids were quantified as themselves.

### GC analysis of free amino acids

The extracts were analysed on a Chrompack CP 9001 instrument (Chrompack, Middelburg, The Netherlands) equipped with a flame ionisation detector (FID), and an automatic liquid sampler (CP-9050, Chrompack) [12]. The amino acids were identified by their retention times and chromatographic comparison with authentic standards. Quantification was based on the internal standard method using L- *p*-chlorophenylalanine.

## Results and discussion

### Phenolic compounds

Quince pulp presented a chemical profile composed by six identified phenolic compounds: 3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic and 3,5-dicaffeoylquinic acids, quercetin 3-galactoside and rutin. Quince peel contained thirteen phenolics: the six compounds presented in pulps, plus kaempferol 3-glucoside, kaempferol 3-rutinoside, and five not totally identified compounds (one kaempferol glycoside, two quercetin glycosides acylated with *p*-coumaric acid and two kaempferol glycosides acylated with *p*-coumaric acid). The samples now under study revealed the same phenolic composition as those collected in 2000 [10].

In pulp, caffeoylquinic acids represented 97% of the determined phenolics, with 3-*O*-caffeoylquinic acid being the most abundant (45%). Peel contained 66% of flavonol derivatives, with rutin being the major one (47%). Peel had a higher amount of phenolics than pulp (about 8 times) (Table 1).

In order to test if it is possible to detect quince peel in jams, two quince jams were prepared, one of them with peeled fruit (jam A) and the other one with unpeeled fruit (jam B). The total flavonoid content of jam A was 3%, while that of jam B was 19%. The amount of the total identified phenolics was duplicated in the jam that was prepared with unpeeled quinces (jam B) (Table 1). As expected, qualitatively, these two jams had different phenolic profiles: jam A, as quince pulp, presented a profile composed by 3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic and 3,5-dicaffeoylquinic acids, quercetin 3-galactoside and rutin, while jam B contained these six compounds, plus kaempferol 3-glucoside, kaempferol 3-rutinoside, and the five not totally identified compounds found in quince peels. So, as previously reported [10], it seems that the phenolic profile determination allows the detection of adulterations in quince jams by addition of quince peel.

Although quince jam A had been prepared with 50% of pulp, its total phenolic content corresponded to about 57% of that of the used pulp. This could be due to evaporation during thermal processing. As in quince pulp, caffeoylquinic acids represented 97% of the determined phenolics in jam A. Nevertheless, the major compound (5-*O*-caffeoylquinic acid) was not the same, which may indicate the occurrence of isomerisation of caffeoylquinic acids .

**Table 1** Phenolic composition of quince pulp, peel and jams (mg/kg). Quantification by external standard technique

| Phenolic compounds | Samples | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Pulp | | Peel | | Jam A | | Jam B | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| 3-CQA | 94.6 | 0.74 | 209.9 | 4.61 | 43.1 | 0.34 | 87.3 | 5.97 |
| 4-CQA | 8.5 | 0.01 | 19.2 | 0.62 | 8.7 | 0.28 | 17.0 | 0.60 |
| 5-CQA | 90.0 | 1.08 | 279.8 | 8.61 | 58.4 | 0.46 | 84.6 | 3.53 |
| 3,5-diCQA | 9.3 | 0.07 | 24.3 | 2.05 | 4.4 | 0.08 | 4.7 | 0.02 |
| Q-3-gal | 2.5 | 0.02 | 60.7 | 5.31 | 1.8 | 0.08 | 5.2 | 0.13 |
| Q-3-rut | 3.3 | 0.04 | 740.8 | 28.14 | 1.9 | 0.06 | 31.1 | 0.73 |
| K-gly | nd | – | 32.2 | 1.57 | nd | – | 0.9 | 0.15 |
| K-3-glu | nd | – | 25.8 | 0.40 | nd | – | 0.7 | 0.04 |
| K-3-rut | nd | – | 51.0 | 1.13 | nd | – | 3.0 | 0.09 |
| Q-gly-*p*-CouA1 | nd | – | 52.0 | 0.92 | nd | – | 1.3 | 0.05 |
| Q-gly-*p*-CouA2 | nd | – | 17.7 | 0.05 | nd | – | 0.6 | 0.02 |
| K-gly-*p*-CouA1 | nd | – | 18.4 | 0.10 | nd | – | 0.9 | 0.03 |
| K-gly-*p*-CouA2 | nd | – | 34.5 | 0.19 | nd | – | 1.0 | 0.15 |
| Σ | 208.1 | | 1566.4 | | 118.3 | | 238.3 | |
| HMF | nd | – | nd | – | 503.9 | 5.52 | 203.0 | 1.64 |

Values are expressed as mean of three determinations
Jam A is quince jam prepared with peeled fruit, Jam B is quince jam prepared with unpeeled fruit
*SD* standard deviation, Σ sum of the determined phenolic compound, *nd* not detected
*3-CQA* 3-*O*-caffeoylquinic acid, *4-CQA* 4-*O*-caffeoylquinic acid, *5-CQA* 5-*O*-caffeoylquinic acid, *3,5-diCQA* 3,5-dicaffeoylquinic acid, *Q-3-Gal* quercetin 3-galactoside, *Q-3-Rut* rutin, *K-Gly* kaempferol glycoside, *K-3-Glu* kaempferol 3-glucoside, *K-3-Rut* kaempferol 3-rutinoside, *Q-gly-p-CouA1* and *Q-gly-p-CouA2* quercetin glycosides acylated with *p*-coumaric acid, *K-gly-p-CouA1* and *K-gly-p-CouA2* kaempferol glycosides acylated with *p*-coumaric acid, *HMF* hydroxymethylfurfural

**Table 2** Organic acid composition of quince pulp, peel and jams (mg/kg). Quantification by external standard technique

| Organic acids | Samples | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Pulp | | Peel | | Jam A | | Jam B | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Oxalic acid | 9.1 | 0.01 | 11.5 | 0.20 | 6.1 | 0.04 | 2.6 | 0.05 |
| Citric acid | 287.5 | 2.81 | 294.3 | 13.88 | 54.0 | 2.02 | 42.1 | 3.56 |
| Ascorbic acid | 159.0 | 6.63 | 189.4 | 9.77 | 34.4 | 0.80 | 49.1 | 1.69 |
| Malic+quinic acids | 6936.2 | 30.12 | 6706.9 | 381.08 | 4457.8 | 144.12 | 4321.3 | 69.38 |
| Shikimic acid | 24.3 | 0.08 | 26.5 | 0.07 | 4.2 | 0.12 | 8.0 | 0.21 |
| Fumaric acid | tr | – | tr | – | tr | – | tr | – |
| Σ | 7416.1 | | 7228.6 | | 4556.4 | | 4423.0 | |

Values are expressed as mean of three determinations
Jam A is quince jam prepared with peeled fruit, Jam B is quince jam prepared with unpeeled fruit
*SD* standard deviation, Σ sum of the determined organic acids, *tr* traces

In chromatograms of quince jams (data not shown) it was possible to observe a peak corresponding to hydroxymethylfurfural (HMF). The presence of this compound is not strange as it results from sugar decomposition by heat during cooking.

## Organic acids

All samples (pulp, peel and jams) presented a similar profile composed of seven identified organic acids: oxalic, citric, ascorbic, malic, quinic, shikimic and fumaric acids.

In quince pulp and peel, the sum of malic acid plus quinic acid represented 93% and all other acids were present in very small amounts, less than 0.5%, with the exceptions of citric and ascorbic acids (about 4 and 2%, respectively). The sum of all quantified acids was approximately 7 g/kg (Table 2), both in pulp and peel, which is in agreement with results previously reported [11].

Due to the similarity between the organic acid profile of pulp and peel, it was not possible, by using this chemical parameter, to differentiate these two parts of the fruit or the jams prepared with peeled or unpeeled fruit.

On comparing the total amount of organic acids in quince jam A with that of the used pulp, it can be seen that it corresponded to approximately 60%, despite being prepared with 50% of pulp. This fact can, probably, be explained by the occurrence of evaporation during jam processing.

In jams, the sum of malic acid plus quinic acid represented 98% and all other acids were present in very small amounts, less than 1.2%. It seems that the high temperatures used during processing caused some destruction of citric, ascorbic and shikimic acids.

**Table 3** Free amino acids composition of quince pulp, peel and jams ($\mu$g/kg). Quantification by internal standard technique

| Amino acids | Samples | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Pulp | | Peel | | Jam A | | Jam B | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Alanine | 16.2 | 0.38 | 12.3 | 0.25 | 16.1 | 0.77 | 23.2 | 0.80 |
| Glycine | 1.4 | 0.05 | 3.5 | 0.15 | 9.4 | 0.23 | 9.1 | 0.20 |
| Valine | 4.1 | 0.16 | 1.7 | 0.27 | 5.1 | 0.37 | 4.7 | 0.23 |
| Leucine | 0.6 | 0.02 | 1.0 | 0.02 | 2.1 | 0.08 | 2.8 | 0.03 |
| Isoleucine | 11.8 | 0.31 | 6.9 | 0.37 | 2.9 | 0.09 | 5.0 | 0.11 |
| Proline | 2.0 | 0.09 | 4.7 | 0.13 | 3.6 | 0.10 | 4.6 | 0.03 |
| Threonine | 10.0 | 0.41 | 15.6 | 0.53 | 35.9 | 0.74 | 45.3 | 1.67 |
| Serine | 33.8 | 0.36 | 77.0 | 0.44 | 16.7 | 0.83 | 30.8 | 1.10 |
| Glutamic acid | 104.9 | 4.76 | 355.5 | 10.17 | 6.0 | 0.24 | 12.6 | 0.20 |
| Asparagine | 22.8 | 0.73 | 8.6 | 0.57 | 160.1 | 1.26 | 112.3 | 1.52 |
| Aspartic acid | 138.5 | 4.92 | 71.2 | 1.75 | 383.4 | 3.13 | 850.7 | 12.15 |
| Methionine | 1.2 | 0.04 | 2.5 | 0.06 | 1.1 | 0.02 | 0.2 | 0.01 |
| Hydroxyproline | 38.2 | 1.33 | 29.7 | 0.53 | 58.7 | 0.94 | 67.9 | 2.82 |
| Phenylalanine | 1.2 | 0.11 | 1.4 | 0.06 | 2.0 | 0.05 | 1.5 | 0.19 |
| Cysteine | 46.9 | 0.68 | 103.9 | 3.07 | 21.7 | 1.14 | 51.7 | 1.73 |
| Glutamine | 12.8 | 0.33 | 24.7 | 0.60 | 4.4 | 0.22 | 7.9 | 0.07 |
| Ornithine | 2.2 | 0.02 | 0.8 | 0.07 | 3.8 | 0.05 | 1.2 | 0.01 |
| Lysine | 8.1 | 0.18 | 10.7 | 0.25 | 5.4 | 0.13 | 8.6 | 0.20 |
| Histidine | 20.1 | 0.73 | 21.2 | 1.43 | 2.3 | 0.15 | 3.1 | 0.10 |
| Tyrosine | 1.3 | 0.04 | 1.1 | 0.03 | 2.1 | 0.04 | 3.4 | 0.06 |
| Tryptophan | 2.2 | 0.02 | 2.0 | 0.03 | 0.1 | 0.00 | 0.1 | 0.01 |
| Σ | 480.4 | | 755.9 | | 742.7 | | 1246.5 | |

Values are expressed as mean of three determinations
Jam A is quince jam prepared with peeled fruit, Jam B is quince jam prepared with unpeeled fruit
*SD* standard deviation, Σ sum of the determined free amino acids

Free amino acids

As expected [12], all samples (quince fruits and jams) presented a similar qualitative profile composed of 21 identified free amino acids. Peel had a higher amount of total free amino acids than pulp (about 1.6 times) (Table 3). The five most abundant free amino acids were: aspartic and glutamic acids, cysteine, serine and hydroxyproline, and corresponded to about 75% and 85% of the totality of free amino acids of pulp and peel, respectively.

The five major free amino acids in jams were aspartic acid, asparagine, hydroxyproline, cysteine and threonine, that together accounted for about 90% of the totality of free amino acids (Table 3). Proline, hydroxyproline, phenylalanine, ornithine and tyrosine percentages seemed to be characteristic of pulp, since they stayed approximately constant in jam A.

Quince jams had higher total free amino acid content than pulp and peel (Table 3), which could be due to hydrolysis of proteins, peptides or other compounds with amino acids in their constitution, which can occur during thermal processing (in acid medium). Glutamic acid, histidine and tryptophan were present in much lower amounts in jams than in pulp or peel, which can be due to their thermolability. These amino acids can also take an active part in Maillard reactions and/or in browning processes after the enzymatic oxidation of polyphenols.

In conclusion, although jam processing leads to caffeoylquinic acid isomerisation and to the degradation of some organic acids (citric, ascorbic and shikimic acids), the total contents of the determined phenolics and organic acids in quince fruit and jam did not indicate appreciable changes. The free amino acid profile was changed by thermal processing, probably due to hydrolysis of amino acid-derived compounds.

The determination of the phenolic profile also allowed detection of quince peel in the jam prepared with unpeeled fruit, but organic acids and free amino acids profiles were not suitable for this purpose.

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# VII. TRATAMENTO ESTATÍSTICO

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# Quince (*Cydonia oblonga* Miller) Fruit Characterization Using Principal Component Analysis

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This paper presents a large amount of data on the composition of quince fruit with regard to phenolic compounds, organic acids, and free amino acids. Subsequently, principal component analysis (PCA) is carried out to characterize this fruit. The main purposes of this study were (i) the clarification of the interactions among three factors—quince fruit part, geographical origin of the fruits, and harvesting year—and the phenolic, organic acid, and free amino acid profiles; (ii) the classification of the possible differences; and (iii) the possible correlation among the contents of phenolics, organic acids, and free amino acids in quince fruit. With these aims, quince pulp and peel from nine geographical origins of Portugal, harvested in three consecutive years, for a total of 48 samples, were studied. PCA was performed to assess the relationship among the different components of quince fruit phenolics, organic acids, and free amino acids. Phenolics determination was the most interesting. The difference between pulp and peel phenolic profiles was more apparent during PCA. Two PCs accounted for 81.29% of the total variability, PC1 (74.14%) and PC2 (7.15%). PC1 described the difference between the contents of caffeoylquinic acids (3-*O*-, 4-*O*-, and 5-*O*-caffeoylquinic acids and 3,5-*O*-dicaffeoylquinic acid) and flavonoids (quercetin 3-galactoside, rutin, kaempferol glycoside, kaempferol 3-glucoside, kaempferol 3-rutinoside, quercetin glycosides acylated with *p*-coumaric acid, and kaempferol glycosides acylated with *p*-coumaric acid). PC2 related the content of 4-*O*-caffeoylquinic acid with the contents of 5-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids. PCA of phenolic compounds enables a clear distinction between the two parts of the fruit. The data presented herein may serve as a database for the detection of adulteration in quince derivatives.

**KEYWORDS:** ***Cydonia oblonga* Miller; quince fruit; pulp; peel; phenolic compounds; organic acids; free amino acids; principal component analysis**

## INTRODUCTION

Quince is the fruit of a deciduous tree of the Rosaceae family, *Cydonia oblonga* Miller. Although quince fruit is not edible raw because of its hardness, bitterness, and astringency, it is very appreciated in Portugal for its jam, called "marmelada". According to Portuguese legislation (*1*), quince jam is the food product of a homogeneous and consistent mixture obtained exclusively by boiling quince mesocarp with sugars.

Before 1998, only a few chemical studies have been developed in this matrix. These works concerned mainly the volatile constituents of quince fruit (*2*−*7*) and the glucosides of procyanidin polymers (*8*).

For the past few years, quince fruit and its derivatives have been studied by our research group to examine their chemical constituents (*9*−*19*) and to evaluate their antioxidant potential (*20*). Among the various studied chemical parameters, the phenolic profile seemed to be the most useful in the discrimination of the different parts of quince fruit (pulp, peel, and seed) (*10*, *14*, *15*). This procedure also allowed the detection of adulterations in quince jams by the addition of quince peel (*10*).

As the published literature was based on results from only one year of quince harvest (2000), and considering the pos-

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**Table 1.** Phenolic Composition of Quince Pulps[a]

| obser-vation | geographical origin | year | phenolic compound (%) 3-CQA | SD | 4-CQA | SD | 5-CQA | SD | 3,5-diCQA | SD | Q-3-Gal | SD | Q-3-Rut | SD | Σ (mg/kg) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Amarante | 2000 | 24.15 | 0.342 | 4.46 | 0.046 | 59.28 | 0.763 | 8.03 | 0.063 | nd | | 4.08 | 0.149 | 134.3 |
| 2 | | 2001 | 28.87 | 0.992 | 17.74 | 0.676 | 50.11 | 1.249 | 2.46 | 0.009 | 0.24 | 0.012 | 0.57 | 0.020 | 167.1 |
| 3 | | 2002 | 45.44 | 0.354 | 4.11 | 0.007 | 43.23 | 0.521 | 4.46 | 0.031 | 1.19 | 0.011 | 1.57 | 0.020 | 208.1 |
| 4 | Baião | 2000 | 16.84 | 0.436 | 2.90 | 0.259 | 69.11 | 0.692 | 5.95 | 0.277 | nd | | 5.20 | 0.580 | 142.2 |
| 5 | | 2001 | 21.12 | 0.349 | 1.73 | 0.070 | 71.55 | 0.947 | 2.51 | 0.124 | nd | | 3.09 | 0.036 | 135.6 |
| 6 | | 2002 | 22.15 | 0.381 | 2.20 | 0.025 | 69.81 | 1.115 | 4.35 | 0.032 | nd | | 1.49 | 0.087 | 364.8 |
| 7 | Bragança | 2000 | 7.63 | 0.045 | 4.87 | 0.014 | 47.80 | 0.776 | tr | | nd | | 39.71 | 0.682 | 11.7 |
| 8 | | 2001 | 32.74 | 0.648 | 2.53 | 0.068 | 62.79 | 0.273 | 1.94 | 0.041 | nd | | nd | | 162.4 |
| 9 | | 2002 | 41.37 | 0.624 | 4.95 | 0.008 | 46.47 | 0.902 | 4.72 | 0.063 | nd | | 2.49 | 0.087 | 160.1 |
| 10 | Caminha | 2001 | 49.10 | 0.547 | 7.32 | 0.103 | 43.58 | 0.298 | tr | | nd | | nd | | 154.1 |
| 11 | Covilhã | 2000 | 22.28 | 0.707 | 3.38 | 0.007 | 69.30 | 0.434 | 2.37 | 0.062 | nd | | 2.66 | 0.093 | 155.9 |
| 12 | | 2001 | 35.84 | 0.014 | 4.08 | 0.183 | 54.32 | 0.926 | 3.51 | 0.087 | nd | | 2.25 | 0.041 | 206.7 |
| 13 | | 2002 | 29.27 | 0.591 | 5.15 | 0.261 | 60.76 | 0.433 | 3.35 | 0.117 | nd | | 1.46 | 0.041 | 260.9 |
| 14 | Custóias | 2001 | 26.07 | 0.254 | 2.72 | 0.235 | 65.83 | 1.922 | 1.93 | 0.023 | 0.89 | 0.021 | 2.57 | 0.053 | 322.9 |
| 15 | | 2002 | 32.41 | 0.816 | 9.72 | 0.236 | 54.34 | 0.029 | 2.63 | 0.040 | 0.30 | 0.001 | 0.59 | 0.006 | 518.6 |
| 16 | Pinhel | 2000 | 20.98 | 0.259 | 2.73 | 0.019 | 69.17 | 0.861 | 2.36 | 0.076 | 1.57 | 0.118 | 3.19 | 0.024 | 268.3 |
| 17 | | 2001 | 37.33 | 0.069 | 2.95 | 0.032 | 57.25 | 0.279 | 2.47 | 0.016 | nd | | nd | | 343.8 |
| 18 | | 2002 | 31.24 | 1.345 | 9.17 | 0.214 | 54.05 | 0.219 | 3.68 | 0.176 | nd | | 1.85 | 0.036 | 365.0 |
| 19 | Vila Real | 2000 | 25.67 | 0.363 | 5.52 | 0.290 | 61.94 | 0.444 | 4.91 | 0.129 | nd | | 1.96 | 0.116 | 88.3 |
| 20 | | 2001 | 33.84 | 0.254 | 3.32 | 0.156 | 55.38 | 0.329 | 2.59 | 0.013 | nd | | 4.87 | 0.604 | 136.5 |
| 21 | | 2002 | 45.17 | 0.288 | 3.05 | 0.002 | 45.24 | 0.156 | 4.02 | 0.048 | 1.08 | 0.011 | 1.43 | 0.065 | 313.2 |
| 22 | Viseu | 2000 | 27.51 | 0.016 | 4.55 | 0.068 | 59.90 | 0.945 | 3.31 | 0.159 | nd | | 4.73 | 0.522 | 109.7 |
| 23 | | 2001 | 44.88 | 0.568 | 6.11 | 0.096 | 42.89 | 0.169 | 3.33 | 0.055 | nd | | 2.78 | 0.163 | 212.8 |
| 24 | | 2002 | 30.28 | 0.507 | 17.77 | 0.062 | 48.50 | 0.660 | 0.49 | 0.015 | nd | | 2.97 | 0.028 | 434.1 |
| mean | | | 30.51 | | 5.54 | | 56.78 | | 3.14 | | 0.22 | | 3.81 | | 224.1 |
| max | | | 49.10 | | 17.77 | | 71.55 | | 8.03 | | 1.57 | | 39.71 | | 518.6 |
| min | | | 7.63 | | 1.73 | | 42.89 | | tr | | nd | | nd | | 11.7 |
| SD | | | 10.058 | | 4.273 | | 9.400 | | 1.802 | | 0.458 | | 7.790 | | 120.53 |

[a] SD, standard deviation of three determinations; nd, not detected; tr, traces; Σ, sum of the determined phenolics; 3-CQA, 3-*O*-caffeoylquinic acid; 4-CQA, 4-*O*-caffeoylquinic acid; 5-CQA, 5-*O*-caffeoylquinic acid; 3,5-CQA, 3,5-*O*-dicaffeoylquinic acid; Q-3-Gal, quercetin 3-galactoside; Q-3-Rut, rutin.

sibility of the influence of geographical origin and harvesting year on the chemical profile, the paper herein reports, for the first time, the phenolic, organic acid, and free amino acid composition of quince fruit harvested in 2001 and 2002. Principal component analysis (PCA) was applied to the results of the three years of quince harvest to determine the relationship among the different components of quince fruit phenolics, organic acids, and free amino acids. PCA and ANOVA were performed separately for each chemical parameter.

The main purposes of this study were (i) to clarify the interactions between the studied factors (quince fruit part, geographical origin of the fruits, and harvesting year) and the phenolic, organic acid, and free amino acid profiles; (ii) to classify the possible differences; and (iii) to verify if there is a correlation among the contents of phenolics, organic acids, and free amino acids in quince fruit. Finally, after the acquisition of these data, we indicate what is the most useful parameter with regard to the quality control of these food products.

## MATERIALS AND METHODS

**Samples.** Healthy quince fruit samples were collected in different places in northern (Amarante, Baião, Vila Real, Bragança, Custóias, and Caminha) and central (Viseu, Pinhel, and Covilhã) Portugal, in 2000 (14 samples), 2001 (18 samples), and 2002 (16 samples). For each sample from each geographical origin, ~1 kg of quince fruits was manually collected from around quince trees present in the quince orchard. All fruits were separated into pulp and peel. Each part of the fruit was cut in thin slices and freeze-dried. Lyophilization was carried out using a Labconco 4.5 apparatus (Kansas City, MO).

**Standards.** The standards were from Sigma (St. Louis, MO) and from Extrasynthése (Genay, France). Methanol and formic and hydrochloric acids were obtained from Merck (Darmstadt, Germany), and sulfuric acid was from Pronalab (Lisboa, Portugal). Ethyl chloroformate (ECF) was from Aldrich (Steinheim, Germany) and pyridine from Fluka (Neu-Ulm, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

**Solid-Phase Extraction (SPE) Columns.** The Isolute C18 non-end-capped (NEC) SPE columns (50 μm particle size, 60 Å porosity; 10 g of sorbent mass/70 mL of reservoir volume) were purchased from International Sorbent Technology Ltd. (Mid Glamorgan, U.K.). The benzenesulfonic SCX Spe-ed SPE cartridges (200 mg; 3 mL) were obtained from Applied Separations (Allentown, PA).

**Extraction and HPLC Analysis of Phenolic Compounds.** The extraction of phenolics was achieved as previously reported (*9*, *10*, *15*, *19*). Briefly, each sample (~1 g) was thoroughly mixed with water (pH 2 with HCl) until complete extraction of the phenolic compounds (negative reaction to 20% NaOH) and filtered. One percent methanol was added to the filtrate, which was then passed through an Isolute C18 (NEC) column, preconditioned with 60 mL of methanol and 140 mL of water (pH 2 with HCl). Sugars and other polar compounds were eluted with the aqueous solvent. The retained phenolic fraction was then eluted with methanol (~50 mL). The extract was concentrated to dryness under reduced pressure (40 °C) and redissolved in methanol (1 mL), and 20 μL was analyzed by HPLC.

Separation of the phenolics was achieved as reported previously (*9*, *10*, *15*–*20*), with an analytical HPLC unit (Gilson), using a Spherisorb ODS2 (25.0 × 0.46 cm; 5 μm, particle size) column. The solvent system used was a gradient of water/formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% B at 42 min, 50% B at 44 min, 55% B at 47 min, 70% B at 50 min, 75% B at 56 min, and 80% B at 60 min, at a solvent flow rate of 0.9 mL/min. Detection was achieved with a Gilson diode array detector. The compounds in each sample were identified by comparing

**Table 2.** Phenolic Composition of Quince Peels[a]

| obser- | geographical | | phenolic compound (%) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| vation | origin | year | 3-CQA | SD | 4-CQA | SD | 5-CQA | SD | 3,5-diCQA | SD | Q-3-Gal | SD | Q-3-Rut | SD | K-3-Gly | SD |
| 25 | Amarante | 2000 | 10.72 | 0.257 | 1.53 | 0.066 | 26.65 | 0.083 | 3.50 | 0.159 | 2.39 | 0.055 | 39.56 | 4.354 | 2.18 | 0.017 |
| 26 | | 2001 | 15.26 | 0.121 | 1.20 | 0.134 | 18.70 | 0.039 | 1.71 | 0.091 | 7.57 | 0.012 | 44.40 | 0.232 | 2.18 | 0.094 |
| 27 | | 2002 | 13.40 | 0.295 | 1.22 | 0.039 | 17.86 | 0.550 | 1.55 | 0.131 | 3.88 | 0.339 | 47.29 | 1.797 | 2.06 | 0.100 |
| 28 | Baião | 2000 | 1.78 | 0.073 | 0.31 | 0.009 | 9.78 | 0.548 | 0.87 | 0.022 | 13.68 | 0.460 | 47.34 | 1.114 | 5.55 | 0.140 |
| 29 | | 2001 | 4.22 | 0.105 | 0.96 | 0.009 | 16.56 | 0.316 | 1.01 | 0.088 | 10.13 | 0.159 | 45.66 | 0.044 | 3.68 | 0.018 |
| 30 | | 2002 | 9.16 | 0.297 | 0.78 | 0.005 | 31.63 | 0.848 | 1.64 | 0.097 | 12.13 | 0.228 | 31.32 | 1.018 | 3.29 | 0.087 |
| 31 | Bragança | 2000 | 0.12 | 0.008 | 0.10 | 0.001 | 2.10 | 0.015 | 0.76 | 0.046 | 12.17 | 0.939 | 61.80 | 0.703 | 8.74 | 0.743 |
| 32 | | 2001 | 7.35 | 0.007 | 2.53 | 0.046 | 26.74 | 0.327 | tr | | 11.03 | 0.105 | 36.52 | 1.442 | 4.20 | 0.056 |
| 33 | Caminha | 2002 | 29.08 | 0.227 | 2.75 | 0.013 | 35.17 | 0.271 | 2.99 | 0.084 | 4.09 | 0.039 | 17.74 | 0.077 | 2.38 | 0.001 |
| 34 | | 2001 | 19.88 | 0.460 | 2.70 | 0.062 | 27.84 | 0.450 | 1.02 | 0.017 | 4.77 | 0.158 | 32.68 | 0.133 | 1.88 | 0.142 |
| 35 | Covilhã | 2000 | 1.21 | 0.020 | 0.26 | 0.001 | 6.24 | 0.100 | 0.61 | 0.087 | 14.76 | 0.146 | 50.21 | 0.779 | 6.44 | 0.041 |
| 36 | | 2001 | 12.27 | 0.010 | 2.43 | 0.002 | 24.20 | 0.377 | 1.89 | 0.053 | 7.36 | 0.050 | 36.31 | 0.129 | 3.42 | 0.028 |
| 37 | Custóias | 2002 | 12.57 | 0.269 | 8.15 | 0.254 | 38.10 | 0.528 | 2.27 | 0.065 | 6.36 | 0.048 | 22.91 | 0.824 | 2.18 | 0.064 |
| 38 | | 2001 | 10.77 | 0.021 | 1.43 | 0.043 | 54.62 | 0.152 | 1.26 | 0.036 | 6.04 | 0.034 | 22.82 | 0.071 | 0.39 | 0.001 |
| 39 | | 2002 | 23.99 | 0.200 | 2.23 | 0.046 | 43.07 | 0.543 | 1.71 | 0.008 | 3.62 | 0.071 | 14.58 | 0.303 | 2.58 | 0.045 |
| 40 | Pinhel | 2000 | 5.39 | 0.175 | 1.09 | 0.043 | 22.98 | 0.916 | 1.21 | 0.034 | 11.01 | 0.324 | 44.40 | 1.284 | 2.33 | 0.050 |
| 41 | | 2001 | 10.49 | 0.117 | 1.40 | 0.078 | 23.45 | 0.151 | 1.51 | 0.130 | 11.69 | 0.188 | 36.43 | 0.883 | 3.06 | 0.104 |
| 42 | | 2002 | 11.06 | 0.011 | 1.11 | 0.005 | 21.43 | 0.080 | 1.36 | 0.015 | 8.85 | 0.272 | 37.40 | 0.056 | 4.49 | 0.097 |
| 43 | Vila Real | 2000 | 12.08 | 0.300 | 1.91 | 0.026 | 26.85 | 0.821 | 2.69 | 0.127 | 8.45 | 0.290 | 39.07 | 1.605 | 1.37 | 0.023 |
| 44 | | 2001 | 6.93 | 0.328 | 3.82 | 0.109 | 17.16 | 0.705 | 1.64 | 0.037 | 10.85 | 0.173 | 40.38 | 0.449 | 3.68 | 0.222 |
| 45 | | 2002 | 18.57 | 0.388 | 1.21 | 0.001 | 24.26 | 0.442 | 2.02 | 0.091 | 8.55 | 0.103 | 33.09 | 0.759 | 2.42 | 0.024 |
| 46 | Viseu | 2000 | 5.33 | 0.081 | 0.83 | 0.028 | 12.67 | 0.133 | 1.29 | 0.060 | tr | | 57.88 | 1.013 | 4.36 | 0.075 |
| 47 | | 2001 | 21.41 | 0.512 | 1.65 | 0.078 | 21.16 | 0.815 | 2.44 | 0.105 | 8.24 | 0.303 | 31.81 | 1.248 | 2.73 | 0.050 |
| 48 | | 2002 | 19.41 | 1.079 | 1.67 | 0.013 | 23.38 | 0.861 | 2.05 | 0.078 | 7.96 | 0.319 | 34.04 | 0.950 | 2.20 | 0.008 |
| mean | | | 11.77 | | 1.80 | | 23.86 | | 1.63 | | 8.15 | | 37.74 | | 3.24 | |
| max | | | 29.08 | | 8.15 | | 54.62 | | 3.50 | | 14.76 | | 61.80 | | 8.74 | |
| min | | | 0.12 | | 0.10 | | 2.10 | | tr | | tr | | 14.58 | | 0.39 | |
| SD | | | 7.426 | | 1.613 | | 11.478 | | 0.787 | | 3.743 | | 11.437 | | 1.773 | |

| obser- | geographical | | phenolic compound (%) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| vation | origin | year | K-3-Glu | SD[a] | K-3-Rut | SD[a] | Q-Gly-pC1 | SD[a] | Q-Gly-pC2 | SD[a] | K-Gly-pC1 | SD[a] | K-Gly-pC2 | SD[a] | Σ (mg/kg) |
| 25 | Amarante | 2000 | 1.53 | 0.055 | 3.38 | 0.056 | 3.08 | 0.081 | 1.20 | 0.073 | 1.57 | 0.130 | 2.71 | 0.206 | 1093.8 |
| 26 | | 2001 | 0.84 | 0.028 | 2.39 | 0.025 | 2.46 | 0.023 | 1.07 | 0.010 | 0.80 | 0.001 | 1.44 | 0.012 | 981.0 |
| 27 | | 2002 | 1.65 | 0.026 | 3.26 | 0.072 | 3.32 | 0.059 | 1.13 | 0.003 | 1.17 | 0.006 | 2.20 | 0.012 | 1566.4 |
| 28 | Baião | 2000 | 5.04 | 0.143 | 7.61 | 0.100 | 1.96 | 0.065 | 1.34 | 0.051 | tr | | 4.74 | 0.208 | 1843.0 |
| 29 | | 2001 | 3.82 | 0.019 | 5.21 | 0.369 | 2.65 | 0.020 | 1.09 | 0.001 | 1.83 | 0.018 | 3.19 | 0.060 | 1417.3 |
| 30 | | 2002 | 2.34 | 0.146 | 3.30 | 0.156 | 1.36 | 0.012 | 0.71 | 0.007 | 0.82 | 0.007 | 1.51 | 0.047 | 1306.2 |
| 31 | Bragança | 2000 | 3.05 | 0.039 | 7.24 | 0.210 | 1.59 | 0.017 | 0.54 | 0.023 | 0.87 | 0.088 | 0.92 | 0.107 | 278.8 |
| 32 | | 2001 | 3.22 | 0.099 | 4.36 | 0.216 | 1.06 | 0.106 | 0.78 | 0.023 | 0.82 | 0.011 | 1.39 | 0.001 | 1173.9 |
| 33 | Caminha | 2002 | 1.84 | 0.011 | 2.39 | 0.002 | 0.45 | 0.012 | 0.18 | 0.003 | 0.32 | 0.003 | 0.62 | 0.011 | 812.3 |
| 34 | | 2001 | 1.02 | 0.033 | 2.02 | 0.074 | 2.55 | 0.008 | 0.82 | 0.009 | 1.80 | 0.013 | 1.03 | 0.020 | 694.6 |
| 35 | Covilhã | 2000 | 4.88 | 0.074 | 6.55 | 0.055 | 2.34 | 0.004 | 1.73 | 0.033 | 1.65 | 0.025 | 3.14 | 0.037 | 935.2 |
| 36 | | 2001 | 2.23 | 0.058 | 4.46 | 0.004 | 1.54 | 0.005 | 0.77 | 0.002 | 1.08 | 0.004 | 2.04 | 0.055 | 758.0 |
| 37 | Custóias | 2002 | 1.41 | 0.091 | 2.39 | 0.111 | 1.20 | 0.081 | 0.47 | 0.002 | 0.88 | 0.081 | 1.11 | 0.038 | 1165.9 |
| 38 | | 2001 | 0.24 | 0.001 | 0.33 | 0.001 | 1.16 | 0.007 | 0.48 | 0.002 | 0.29 | 0.005 | 0.17 | 0.001 | 1284.9 |
| 39 | | 2002 | 2.07 | 0.009 | 2.75 | 0.039 | 0.68 | 0.014 | 0.34 | 0.018 | 0.77 | 0.026 | 1.61 | 0.020 | 632.4 |
| 40 | Pinhel | 2000 | 2.22 | 0.038 | 4.05 | 0.069 | 1.85 | 0.027 | 0.85 | 0.037 | 0.74 | 0.009 | 1.87 | 0.011 | 1882.8 |
| 41 | | 2001 | 2.81 | 0.075 | 3.45 | 0.142 | 2.19 | 0.082 | 0.92 | 0.026 | 0.88 | 0.072 | 1.73 | 0.137 | 1962.4 |
| 42 | | 2002 | 3.90 | 0.193 | 5.82 | 0.284 | 1.12 | 0.037 | 0.55 | 0.003 | 1.04 | 0.016 | 1.86 | 0.063 | 1695.6 |
| 43 | Vila Real | 2000 | 0.91 | 0.024 | 2.29 | 0.090 | 1.58 | 0.046 | 0.81 | 0.009 | 0.54 | 0.002 | 1.45 | 0.004 | 571.3 |
| 44 | | 2001 | 3.05 | 0.022 | 4.91 | 0.120 | 2.18 | 0.028 | 1.39 | 0.008 | 1.41 | 0.108 | 2.60 | 0.241 | 1382.8 |
| 45 | | 2002 | 1.65 | 0.021 | 3.01 | 0.064 | 1.90 | 0.127 | 0.77 | 0.080 | 0.77 | 0.093 | 1.77 | 0.162 | 1118.5 |
| 46 | Viseu | 2000 | 3.50 | 0.043 | 7.50 | 0.108 | 1.81 | 0.017 | 0.93 | 0.020 | 1.34 | 0.080 | 2.56 | 0.001 | 1062.0 |
| 47 | | 2001 | 1.80 | 0.074 | 3.23 | 0.088 | 2.01 | 0.072 | 0.91 | 0.040 | tr | | 2.61 | 0.113 | 1105.7 |
| 48 | | 2002 | 1.56 | 0.037 | 2.97 | 0.056 | 1.81 | 0.052 | 0.67 | 0.012 | 0.74 | 0.010 | 1.53 | 0.051 | 1517.0 |
| mean | | | 2.36 | | 3.95 | | 1.83 | | 0.85 | | 0.92 | | 1.91 | | 1176.7 |
| max | | | 5.04 | | 7.61 | | 3.32 | | 1.73 | | 1.83 | | 4.74 | | 1962.4 |
| min | | | 0.24 | | 0.33 | | 0.45 | | 0.18 | | tr | | 0.17 | | 278.8 |
| SD | | | 1.249 | | 1.890 | | 0.708 | | 0.354 | | 0.503 | | 0.968 | | 435.40 |

[a] SD, standard deviation of three determinations; tr, traces; Σ, sum of the determined phenolics; 3-CQA, 3-*O*-caffeoylquinic acid; 4-CQA, 4-*O*-caffeoylquinic acid; 5-CQA, 5-*O*-caffeoylquinic acid; 3,5-CQA, 3,5-*O*-dicaffeoylquinic acid; Q-3-Gal, quercetin 3-galactoside; Q-3-Rut, rutin; K-3-Gly, kaempferol 3-glycoside; K-3-Glu, kaempferol 3-glucoside; K-3-Rut, kaempferol 3-rutinoside; Q-Gly-pC1 and Q-Gly-pC2, quercetin glycosides acylated with *p*-coumaric acid; K-Gly-pC1 and K-Gly-pC2, kaempferol glycosides acylated with *p*-coumaric acid.

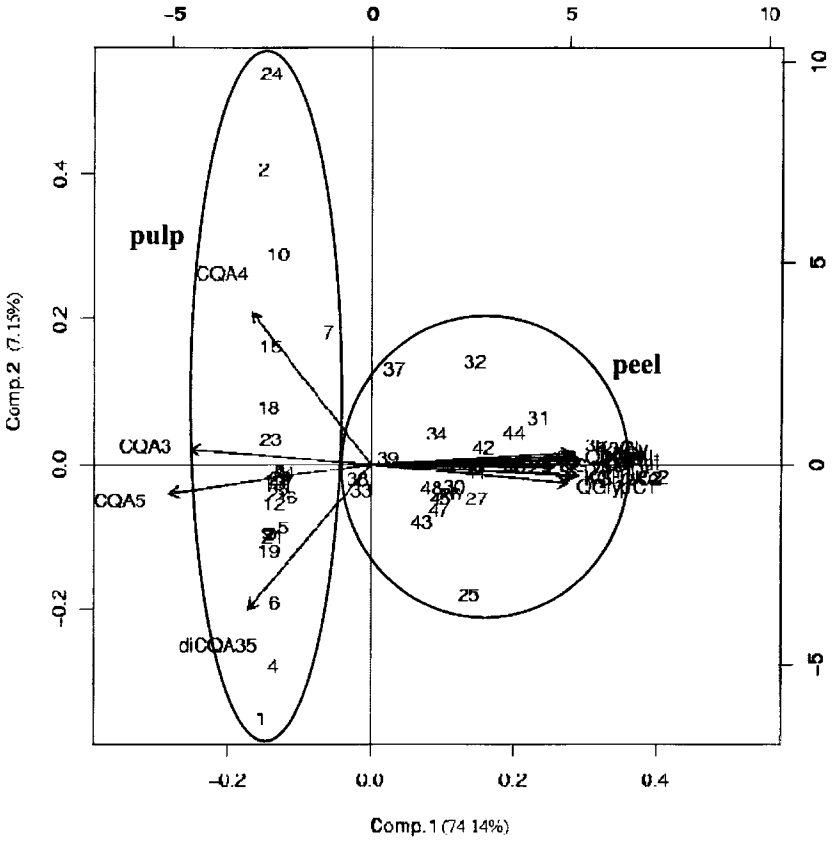


**Figure 1.** PCA of phenolic compounds in quince fruit, from 48 independent observations. CQA3, 3-*O*-caffeoylquinic acid; CQA4, 4-*O*-caffeoylquinic acid; CQA5, 5-*O*-caffeoylquinic acid; diCQA35, 3,5-*O*-dicaffeoylquinic acid; Q3Gal, quercetin 3-galactoside; Q3Rut, rutin; K3Gly, kaempferol 3-glycoside; K3Glu, kaempferol 3-glucoside; K3Rut, kaempferol 3-rutinoside; QGlypC1 and QGlypC2, quercetin glycosides acylated with *p*-coumaric acid; KGlypC1 and KGlypC2, kaempferol glycosides acylated with *p*-coumaric acid.

their retention times and UV−vis spectra in the 200−400 nm range with the library of spectra previously compiled by the authors. Peak purity was checked by means of the Gilson 160 SpectraViewer Software Contrast Facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3- and 4-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid. Kaempferol glycoside and kaempferol glycosides acylated with *p*-coumaric acid were quantified as kaempferol 3-glucoside. Quercetin glycosides acylated with *p*-coumaric acid were quantified as quercetin 3-galactoside. The other compounds were quantified as themselves.

**Extraction and HPLC Analysis of Organic Acids.** The sample preparation was performed as reported by Silva et al. (*11*, *15*, *19*). Briefly, each sample (~1 g) was thoroughly mixed with methanol (10 × 50 mL) (40 °C). The methanolic extract was filtered, concentrated to dryness under reduced pressure (40 °C), and redissolved in acid water (pH 2 with HCl) (~50 mL). The aqueous solution was then passed through an Isolute C18 (NEC) column, previously conditioned with 30 mL of methanol and 70 mL of acid water (pH 2 with HCl). The aqueous extract was evaporated to dryness under reduced pressure (40 °C) and redissolved in sulfuric acid 0.01 N (5 mL), and 20 $\mu$L was analyzed by HPLC.

The separation was carried out as previously reported (*11*, *15*, *19*, *20*), with an analytical HPLC unit (Gilson), using a ion exclusion column Nucleogel Ion 300 OA (300 × 7.7 mm), in conjunction with a column heating device at 30 °C. Elution was carried out at a solvent flow rate of 0.1 mL/min, isocratically with 0.01 N sulfuric acid as the mobile phase. Detection was performed with an Gilson UV detector at 214 nm.

Organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Malic and quinic acids were quantified together and as malic acid. The other acids were quantified as themselves.

**Extraction and GC Analysis of Free Amino Acids.** Extraction was conducted according to the method of Silva et al. (*12*, *13*, *15*, *19*). Briefly, each sample (~1.5 g) was thoroughly mixed with 3 × 25 mL of acid water (pH 2.2 with 0.1 M HCl) at room temperature with magnetic stirring for 3 × 10 min. The extracts were gathered, filtered, and passed through an SCX cartridge, previously conditioned with 10 mL of methanol and 10 mL of 5 mM HCl. The amino acids were eluted with a mixture of ammonia (4 M) and methanol (50:50 v/v) (3 × 500 $\mu$L). To each extract, an amount of 150 $\mu$L of L-*p*-chlorophenylalanine solution (10 $\mu$L/mL) (internal standard) was added. The obtained solutions were dried under a $N_2$ stream and kept below 0 °C until derivatization.

The derivatization of L-amino acids was carried out as reported previously (*12*, *13*, *15*, *19*): each dried residue was dissolved in 60 $\mu$L of water and 40 $\mu$L of ethanol/pyridine (4:1), an amount of 5 $\mu$L of ethyl chloroformate was added, and the solution was vortex-mixed (3−5 s). Five minutes later, 150 $\mu$L of dichloromethane and ~0.01 g of NaCl were added, and the vial was thoroughly shaken for the extraction of the derivatives into the organic layer. This phase was transferred into a 200 $\mu$L insert adjustable to the liquid sampler vials. About 1.5 $\mu$L was injected into the gas chromatographic system.

Separation of L-amino acids was achieved by gas chromatography, carried out with a Chrompack CP 9001 instrument (Chrompack, Middelburg, The Netherlands), equipped with a flame ionization detector (FID), and an automatic liquid sampler (CP-9050, Chrompack) (*12*, *13*, *15*, *19*). The injector and the detector were kept at 250 and

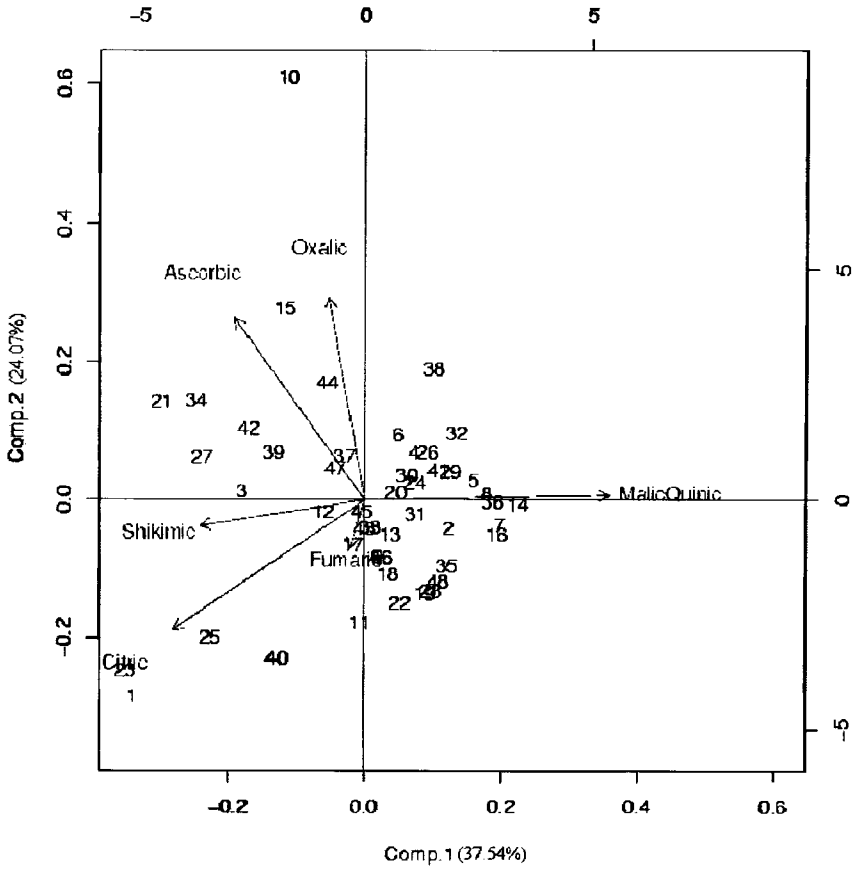


**Figure 2.** PCA of organic acids in quince fruit, from 48 independent observations.

280 °C, respectively. The GC was equipped with an electronic pressure control, allowing programmable gas pressure during the chromatographic run. Helium as carrier gas was used with the following pressure program: increase from initial 50 (1 min hold) to 70 kPa at 4 min. A CP-Sil 19 CB (10 m × 0.25 mm i.d.) WCOT fused-silica capillary column (Varian) was used with the following temperature program: increase from 140 °C (1 min hold) to 280 °C at 40 °C/min.

The amino acids were identified by their retention times and chromatographic comparison with authentic standards. Quantification was based on the internal standard method using l-*p*-chlorophenylalanine.

**Statistical Analysis.** *Experimental Design.* Quince pulp and peel were analyzed in terms of phenolics, organic acids, and free amino acids. The analysis comprised results from nine different locations from Portugal, throughout the harvesting years of 2000, 2001, and 2002. It was not possible to obtain quince fruit samples for every harvesting year. Therefore, the analysis was carried out with the partial factorial design without replication (*21*, *22*), totaling 48 samples. The factors that were evaluated were quince fruit part, geographical origin, and harvesting year. Factor combinations and responses are presented in **Tables 1**−**6**.

*Software.* All statistical analyses involving the experimental data were performed using R 1.9.0 for Linux (*23*).

*Multifactor ANOVA.* A multifactor ANOVA (without replication) was performed to evaluate the effects of the studied factors—quince fruit parts (pulp and peel), Portugal region (Amarante, Baião, Bragança, Caminha, Covilhã, Custóias, Pinhel, Vila Real, and Viseu), and harvesting year (2000−2002)—on phenolics, organic acids, and free amino acids.

The multifactor linear regression model was analyzed for residuals normality and skewness to assess the validity of the ANOVA analysis. Despicable factor effects were removed from the full linear model to improve the accuracy of the analysis. The ANOVA tables and factor probabilities and their combinations were obtained. The Tukey multicomparison test was used to perform pairwise comparisons among factor level means (*24*).

*Correlations.* Pearson correlation coefficients among phenolics, organic acids, and free amino acids were calculated to obtain the possible correlation among the different quince fruit constituents (*24*).

*Principal Component Analysis.* PCA was performed to assess the correspondences among the different components of quince fruit phenolics, organic acids, and free amino acids. PCA was performed separately for each chemical parameter studied (phenolic, organic acid, and free amino acid profiles) and also for the global data.

Principal components (PCs) were analyzed for their variance percentage and component coefficients, to determine their significance. The Gabriel plot (biplot), using optimal scaling, was performed to gain greater insight into the relationships between quince fruit components, to interpret the different groups of data (*25*).

## RESULTS AND DISCUSSION

The analytical variation of the used methodologies is despicable, once these techniques were previously validated (*9*−*12*).

**Phenolic Compounds.** Generally, quince pulps presented a chemical profile composed of six identified phenolic compounds: 3-*O*-, 4-*O*-, and 5-*O*-caffeoylquinic acids, 3,5-*O*-dicaffeoylquinic acid, quercetin 3-galactoside, and rutin (**Table 1**), which is in accordance with previous studies (*10*, *19*, *20*). Usually, quince peels contained 13 phenolics: the 6 compounds present in pulps, plus kaempferol 3-glucoside, kaempferol 3-rutinoside, and 5 not totally identified compounds (1 kaempferol glycoside, 2 quercetin glycosides acylated with *p*-coumaric

**Table 3.** Organic Acid Composition of Quince Pulps[a]

| obser- | geographical | | organic acid (%) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| vation | origin | year | OA | SD | CA | SD | AA | SD | MA + QA | SD | SA | SD | FA | SD | Σ (mg/kg) |
| 1 | Amarante | 2000 | nd | | 8.42 | 0.141 | 0.80 | 0.011 | 90.46 | 0.127 | 0.32 | 0.004 | tr | | 8162.3 |
| 2 | | 2001 | 0.09 | 0.001 | 1.71 | 0.016 | 0.30 | 0.013 | 97.78 | 1.262 | 0.13 | 0.002 | tr | | 10536.6 |
| 3 | | 2002 | 0.12 | 0.001 | 3.88 | 0.038 | 2.14 | 0.089 | 93.53 | 0.406 | 0.33 | 0.001 | tr | | 7416.1 |
| 4 | **Baião** | 2000 | 0.04 | 0.001 | tr | | 2.29 | 0.080 | 97.45 | 1.968 | 0.22 | 0.002 | 0.01 | 0.001 | 6901.4 |
| 5 | | 2001 | 0.08 | 0.001 | 0.35 | 0.010 | 0.93 | 0.005 | 98.50 | 1.353 | 0.14 | 0.002 | nd | | 10670.0 |
| 6 | | 2002 | 0.15 | 0.004 | 0.48 | 0.034 | 1.76 | 0.001 | 97.35 | 1.097 | 0.26 | 0.001 | 0.01 | 0.001 | 5337.1 |
| 7 | Bragança | 2000 | nd | | 0.11 | 0.003 | 0.91 | 0.042 | 98.88 | 1.044 | 0.09 | 0.001 | 0.01 | 0.001 | 12786.4 |
| 8 | | 2001 | 0.06 | 0.002 | 0.34 | 0.003 | 0.79 | 0.010 | 98.69 | 0.613 | 0.12 | 0.001 | nd | | 17393.9 |
| 9 | | 2002 | 0.02 | 0.001 | 1.67 | 0.052 | 0.73 | 0.017 | 97.20 | 0.389 | 0.38 | 0.007 | nd | | 5860.7 |
| 10 | Caminha | 2001 | 0.67 | 0.009 | 0.43 | 0.011 | 3.91 | 0.028 | 94.77 | 0.245 | 0.22 | 0.001 | nd | | 4794.1 |
| 11 | Covilhã | 2000 | 0.04 | 0.001 | 4.00 | 0.091 | 0.11 | 0.014 | 95.68 | 2.225 | 0.17 | 0.002 | 0.01 | 0.001 | 13962.1 |
| 12 | | 2001 | 0.27 | 0.001 | 3.91 | 0.123 | 0.46 | 0.001 | 95.15 | 1.843 | 0.19 | 0.001 | 0.02 | 0.001 | 7397.0 |
| 13 | | 2002 | 0.05 | 0.002 | 1.42 | 0.022 | 0.77 | 0.004 | 97.41 | 1.441 | 0.35 | 0.011 | nd | | 6027.9 |
| 14 | **Custóias** | 2001 | 0.10 | 0.003 | 0.26 | 0.013 | 0.07 | 0.003 | 99.46 | 3.896 | 0.11 | 0.002 | nd | | 8219.0 |
| 15 | | 2002 | 0.22 | 0.013 | 0.95 | 0.047 | 3.88 | 0.087 | 94.65 | 0.096 | 0.30 | 0.005 | nd | | 3497.0 |
| 16 | **Pinhel** | 2000 | 0.05 | 0.003 | 0.39 | 0.006 | 0.32 | 0.013 | 99.10 | 0.711 | 0.14 | 0.001 | 0.01 | 0.001 | 14185.8 |
| 17 | | 2001 | 0.12 | 0.003 | 3.32 | 0.105 | 0.49 | 0.018 | 95.85 | 2.257 | 0.23 | 0.001 | nd | | 6924.9 |
| 18 | | 2002 | nd | | 1.65 | 0.008 | 0.53 | 0.018 | 97.44 | 0.157 | 0.38 | 0.001 | nd | | 3293.0 |
| 19 | Vila Real | 2000 | tr | | 2.13 | 0.018 | 0.34 | 0.008 | 97.35 | 0.573 | 0.17 | 0.001 | 0.01 | 0.001 | 11284.4 |
| 20 | | 2001 | 0.12 | 0.004 | 1.42 | 0.034 | 0.88 | 0.009 | 97.30 | 5.666 | 0.29 | 0.005 | nd | | 6532.8 |
| 21 | | 2002 | 0.21 | 0.001 | 4.39 | 0.015 | 3.72 | 0.064 | 91.40 | 0.708 | 0.28 | 0.001 | 0.01 | 0.001 | 4346.5 |
| 22 | **Viseu** | 2000 | nd | | 2.62 | 0.024 | 0.39 | 0.008 | 96.80 | 0.665 | 0.19 | 0.001 | 0.01 | 0.001 | 9690.1 |
| 23 | | 2001 | 0.06 | 0.002 | 6.37 | 0.155 | 0.56 | 0.052 | 92.31 | 1.325 | 0.68 | 0.001 | 0.01 | 0.001 | 2295.8 |
| 24 | | 2002 | 0.04 | 0.002 | 0.45 | 0.003 | 1.53 | 0.119 | 97.69 | 0.692 | 0.28 | 0.001 | nd | | 8367.2 |
| mean | | | 0.10 | | 2.11 | | 1.19 | | 96.34 | | 0.25 | | 0.00 | | 8161.8 |
| max | | | 0.67 | | 8.42 | | 3.91 | | 99.46 | | 0.68 | | 0.02 | | 17393.9 |
| min | | | nd | | tr | | 0.07 | | 90.46 | | 0.09 | | nd | | 2295.8 |
| SD | | | 0.141 | | 2.157 | | 1.174 | | 2.431 | | 0.127 | | 0.006 | | 3794.32 |

[a] SD, standard deviation of three determinations; nd, not detected; tr, traces; Σ, sum of the determined organic acids; OA, oxalic acid; CA, citric acid; AA, ascorbic acid; MA, malic acid; QA, quinic acid; SA, shikimic acid; FA, fumaric acid.

**Table 4.** Organic Acid Composition of Quince Peels[a]

| obser- | geographical | | organic acid (%) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| vation | origin | year | OA | SD | CA | SD | AA | SD | MA + QA | SD | SA | SD | FA | SD | Σ (mg/kg) |
| 25 | Amarante | 2000 | nd | | 4.65 | 0.134 | 1.00 | 0.018 | 93.80 | 0.023 | 0.55 | 0.002 | 0.01 | 0.001 | 4271.0 |
| 26 | | 2001 | 0.12 | 0.003 | 0.73 | 0.020 | 1.36 | 0.129 | 97.60 | 1.182 | 0.19 | 0.001 | tr | | 8987.8 |
| 27 | | 2002 | 0.16 | 0.003 | 4.07 | 0.192 | 2.62 | 0.135 | 92.78 | 5.272 | 0.37 | 0.001 | tr | | 7228.6 |
| 28 | **Baião** | 2000 | tr | | 1.99 | 0.029 | 0.32 | 0.026 | 97.51 | 1.976 | 0.17 | 0.003 | 0.01 | 0.001 | 13511.1 |
| 29 | | 2001 | 0.14 | 0.001 | 0.87 | 0.037 | 0.74 | 0.050 | 98.08 | 4.163 | 0.17 | 0.001 | nd | | 12185.6 |
| 30 | | 2002 | nd | | 0.54 | 0.048 | 2.11 | 0.108 | 97.14 | 0.368 | 0.22 | 0.001 | nd | | 4676.7 |
| 31 | Bragança | 2000 | tr | | 0.62 | 0.042 | 1.60 | 0.027 | 97.53 | 4.361 | 0.24 | 0.002 | 0.01 | 0.001 | 7757.9 |
| 32 | | 2001 | 0.18 | 0.002 | 0.48 | 0.024 | 1.00 | 0.024 | 98.19 | 0.322 | 0.15 | 0.001 | nd | | 12583.4 |
| 33 | | 2002 | 0.10 | 0.001 | 1.27 | 0.015 | 0.48 | 0.039 | 97.66 | 1.280 | 0.49 | 0.001 | nd | | 5147.5 |
| 34 | Caminha | 2001 | nd | | 1.75 | 0.143 | 4.76 | 0.258 | 93.03 | 2.892 | 0.46 | 0.027 | nd | | 3859.7 |
| 35 | Covilhã | 2000 | tr | | 1.00 | 0.007 | 0.53 | 0.026 | 98.23 | 0.009 | 0.23 | 0.005 | 0.01 | 0.001 | 13974.6 |
| 36 | | 2001 | 0.13 | 0.001 | 0.55 | 0.016 | tr | | 99.15 | 2.659 | 0.17 | 0.001 | nd | | 13413.7 |
| 37 | | 2002 | 0.21 | 0.002 | 1.94 | 0.026 | 1.07 | 0.104 | 96.42 | 1.725 | 0.36 | 0.003 | nd | | 7001.1 |
| 38 | Custóias | 2001 | 0.27 | 0.003 | 0.37 | 0.013 | 1.45 | 0.018 | 97.74 | 3.178 | 0.16 | 0.007 | nd | | 15511.9 |
| 39 | | 2002 | nd | | 1.55 | 0.001 | 3.37 | 0.124 | 94.68 | 0.127 | 0.40 | 0.001 | nd | | 4125.5 |
| 40 | **Pinhel** | 2000 | nd | | 5.87 | 0.738 | 0.57 | 0.012 | 93.40 | 3.248 | 0.15 | 0.002 | 0.01 | 0.001 | 13496.8 |
| 41 | | 2001 | 0.11 | 0.001 | 0.36 | 0.001 | 0.98 | 0.099 | 98.28 | 4.379 | 0.27 | 0.003 | nd | | 10414.5 |
| 42 | | 2002 | 0.21 | 0.001 | 2.67 | 0.043 | 2.45 | 0.060 | 94.27 | 0.664 | 0.40 | 0.002 | 0.01 | 0.001 | 2533.7 |
| 43 | Vila Real | 2000 | 0.13 | 0.006 | 1.01 | 0.066 | 1.64 | 0.022 | 96.87 | 3.779 | 0.28 | 0.011 | 0.06 | 0.001 | 9160.4 |
| 44 | | 2001 | 0.17 | 0.004 | 0.76 | 0.044 | 2.94 | 0.013 | 95.81 | 1.635 | 0.31 | 0.003 | 0.01 | 0.001 | 8203.4 |
| 45 | | 2002 | nd | | 1.64 | 0.092 | 1.85 | 0.034 | 96.23 | 0.105 | 0.27 | 0.003 | nd | | 8276.0 |
| 46 | **Viseu** | 2000 | nd | | 1.89 | 0.052 | 1.18 | 0.016 | 96.67 | 1.630 | 0.25 | 0.001 | 0.01 | 0.001 | 10769.3 |
| 47 | | 2001 | 0.10 | 0.001 | 0.34 | 0.005 | 1.59 | 0.040 | 97.38 | 0.839 | 0.60 | 0.006 | nd | | 4252.0 |
| 48 | | 2002 | nd | | 0.95 | 0.057 | tr | | 98.68 | 1.815 | 0.37 | 0.013 | nd | | 8182.0 |
| mean | | | 0.08 | | 1.58 | | 1.48 | | 96.55 | | 0.30 | | 0.01 | | 8730.2 |
| max | | | 0.27 | | 5.87 | | 4.76 | | 99.15 | | 0.60 | | 0.06 | | 15511.9 |
| min | | | nd | | 0.34 | | tr | | 92.78 | | 0.15 | | nd | | 2533.7 |
| SD | | | 0.088 | | 1.437 | | 1.128 | | 1.893 | | 0.131 | | 0.012 | | 3813.83 |

[a] SD, standard deviation of three determinations; nd, not detected; tr, traces; Σ, sum of the determined organic acids; OA, oxalic acid; CA, citric acid; AA, ascorbic acid; MA, malic acid; QA, quinic acid; SA, shikimic acid; FA, fumaric acid.

**Table 5.** Free Amino Acid Composition of Quince Pulps[a]

| observation | geographical origin | year | amino acid (%) Ala | SD | Gly | SD | Val | SD | Leu | SD | Ile | SD | Pro | SD | Thr | SD | Ser | SD | Glu | SD | Asn | SD | Asp | SD[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Amarante | 2000 | 2.45 | 0.196 | 12.88 | 0.540 | 2.61 | 0.116 | 0.91 | 0.030 | 2.61 | 0.050 | 0.95 | 0.040 | 0.50 | 0.042 | 1.86 | 0.145 | 7.93 | 0.491 | 17.23 | 0.517 | 15.06 | 0.989 |
| 2 | | 2001 | 1.94 | 0.086 | 7.92 | 0.269 | 0.29 | 0.009 | 0.16 | 0.009 | 0.42 | 0.024 | 0.22 | 0.009 | 3.33 | 0.058 | 1.44 | 0.047 | 20.67 | 0.471 | 15.50 | 0.547 | 21.97 | 1.176 |
| 3 | | 2002 | 3.38 | 0.079 | 0.29 | 0.011 | 0.85 | 0.033 | 0.13 | 0.004 | 2.46 | 0.063 | 0.42 | 0.018 | 2.09 | 0.085 | 7.03 | 0.075 | 21.84 | 0.992 | 4.75 | 0.151 | 28.84 | 1.024 |
| 4 | Bairão | 2000 | 5.96 | 0.196 | 17.03 | 0.540 | 4.58 | 0.116 | 1.51 | 0.103 | 6.72 | 0.285 | 1.50 | 0.057 | 3.60 | 0.067 | 7.46 | 0.622 | 8.11 | 0.123 | 12.50 | 0.341 | 15.67 | 0.548 |
| 5 | | 2001 | 1.00 | 0.011 | 46.69 | 0.747 | 0.22 | 0.005 | 0.08 | 0.002 | 1.80 | 0.024 | 0.14 | 0.007 | 1.44 | 0.036 | 0.83 | 0.065 | 10.62 | 0.257 | 9.72 | 0.461 | 13.79 | 0.304 |
| 6 | | 2002 | 3.77 | 0.140 | 16.64 | 0.538 | 0.83 | 0.025 | 0.18 | 0.003 | 1.23 | 0.022 | 0.23 | 0.010 | 1.13 | 0.050 | 1.12 | 0.027 | 17.98 | 0.207 | 5.92 | 0.171 | 22.58 | 0.075 |
| 7 | Bragança | 2000 | 1.60 | 0.048 | 13.70 | 0.539 | 1.80 | 0.058 | 0.47 | 0.030 | 1.21 | 0.058 | 1.06 | 0.036 | 0.95 | 0.050 | 1.82 | 0.021 | 4.46 | 0.046 | 17.88 | 0.555 | 23.01 | 0.216 |
| 8 | | 2001 | 1.24 | 0.037 | 32.61 | 1.545 | 0.96 | 0.039 | 0.08 | 0.002 | 1.96 | 0.039 | 0.15 | 0.007 | 1.42 | 0.065 | 1.93 | 0.049 | 10.56 | 0.260 | 18.73 | 0.707 | 9.06 | 0.253 |
| 9 | | 2002 | 1.75 | 0.034 | 8.06 | 0.252 | 1.02 | 0.020 | 0.14 | 0.004 | 0.69 | 0.016 | 0.21 | 0.003 | 0.96 | 0.003 | 1.79 | 0.054 | 10.76 | 0.182 | 40.04 | 0.245 | 11.34 | 0.107 |
| 10 | Caminha | 2001 | 0.95 | 0.013 | 0.15 | 0.006 | 0.27 | 0.013 | 0.04 | 0.001 | 0.28 | 0.017 | 0.11 | 0.005 | 0.27 | 0.004 | 0.52 | 0.008 | 2.82 | 0.098 | 55.72 | 1.031 | 20.03 | 0.330 |
| 11 | Covilhã | 2000 | 2.44 | 0.094 | 0.83 | 0.036 | 0.78 | 0.028 | 0.35 | 0.023 | 2.01 | 0.125 | 0.58 | 0.028 | 2.53 | 0.142 | 8.91 | 0.119 | 9.45 | 0.415 | 9.44 | 0.063 | 32.25 | 1.753 |
| 12 | | 2001 | 1.48 | 0.053 | 0.47 | 0.012 | 0.48 | 0.014 | 0.09 | 0.002 | 0.61 | 0.020 | 0.46 | 0.025 | 5.66 | 0.260 | 2.18 | 0.051 | 5.42 | 0.226 | 50.32 | 1.814 | 20.37 | 0.029 |
| 13 | | 2002 | 1.47 | 0.036 | 3.93 | 0.047 | 0.71 | 0.018 | 0.12 | 0.002 | 0.31 | 0.005 | 0.18 | 0.003 | 0.33 | 0.013 | 0.49 | 0.007 | 9.77 | 0.050 | 45.70 | 0.861 | 15.86 | 0.337 |
| 14 | Custóias | 2001 | 0.61 | 0.009 | 3.86 | 0.092 | 0.84 | 0.009 | 0.09 | 0.002 | 0.38 | 0.006 | 0.23 | 0.003 | 2.66 | 0.039 | 1.33 | 0.070 | 7.68 | 0.230 | 27.58 | 0.353 | 22.01 | 0.197 |
| 15 | | 2002 | 1.68 | 0.002 | 2.95 | 0.074 | 0.28 | 0.001 | 0.04 | 0.001 | 0.12 | 0.003 | 0.22 | 0.006 | 0.99 | 0.003 | 0.51 | 0.018 | 11.59 | 0.414 | 30.55 | 0.492 | 18.43 | 0.150 |
| 16 | Pinhel | 2000 | 3.59 | 0.103 | 3.47 | 0.211 | 1.55 | 0.080 | 0.49 | 0.008 | 1.56 | 0.042 | 0.65 | 0.033 | 0.76 | 0.023 | 2.44 | 0.121 | 12.29 | 0.859 | 8.95 | 0.262 | 33.97 | 1.139 |
| 17 | | 2001 | 2.04 | 0.002 | 44.98 | 1.648 | 1.31 | 0.060 | 0.27 | 0.007 | 2.14 | 0.095 | 0.14 | 0.004 | 1.16 | 0.046 | 0.92 | 0.074 | 12.97 | 0.585 | 8.28 | 0.151 | 15.05 | 0.905 |
| 18 | | 2002 | 3.32 | 0.046 | 4.53 | 0.173 | 2.70 | 0.116 | 0.41 | 0.008 | 0.98 | 0.033 | 0.44 | 0.013 | 0.84 | 0.031 | 4.99 | 0.201 | 20.57 | 0.510 | 28.63 | 0.945 | 13.42 | 0.355 |
| 19 | Vila Real | 2000 | 7.93 | 0.301 | 5.22 | 0.169 | 1.73 | 0.077 | 0.49 | 0.020 | 1.84 | 0.008 | 2.43 | 0.061 | 1.16 | 0.048 | 5.11 | 0.293 | 5.59 | 0.131 | 39.78 | 0.821 | 11.01 | 0.412 |
| 20 | | 2001 | 2.17 | 0.023 | 17.86 | 0.433 | 0.71 | 0.007 | 0.13 | 0.007 | 0.96 | 0.032 | 0.31 | 0.010 | 0.39 | 0.038 | 1.79 | 0.097 | 10.85 | 0.176 | 31.99 | 1.107 | 14.89 | 0.464 |
| 21 | | 2002 | 1.46 | 0.067 | 2.08 | 0.070 | 0.43 | 0.013 | 0.10 | 0.002 | 0.39 | 0.013 | 0.34 | 0.005 | 0.57 | 0.010 | 3.72 | 0.020 | 20.83 | 0.462 | 22.40 | 0.811 | 17.71 | 0.767 |
| 22 | Viseu | 2000 | 3.97 | 0.143 | 2.03 | 0.093 | 1.82 | 0.073 | 0.40 | 0.015 | 1.93 | 0.057 | 1.05 | 0.043 | 2.30 | 0.015 | 4.57 | 0.068 | 3.62 | 0.219 | 12.76 | 0.670 | 35.21 | 0.143 |
| 23 | | 2001 | 1.38 | 0.164 | 1.69 | 0.065 | 0.49 | 0.043 | 0.19 | 0.010 | 0.24 | 0.007 | 0.25 | 0.009 | 2.42 | 0.045 | 0.70 | 0.010 | 12.79 | 0.276 | 28.30 | 1.341 | 26.31 | 0.839 |
| 24 | | 2002 | 1.43 | 0.040 | 10.88 | 0.231 | 0.47 | 0.013 | 0.08 | 0.001 | 0.59 | 0.021 | 0.24 | 0.004 | 0.12 | 0.006 | 2.75 | 0.045 | 20.75 | 0.330 | 14.84 | 0.182 | 17.73 | 0.374 |
| mean | | | 2.46 | | 10.86 | | 1.16 | | 0.29 | | 1.39 | | 0.52 | | 1.49 | | 2.76 | | 11.66 | | 23.23 | | 19.82 | |
| max | | | 7.93 | | 46.69 | | 4.58 | | 1.51 | | 6.72 | | 2.43 | | 5.66 | | 8.91 | | 21.84 | | 55.72 | | 35.21 | |
| min | | | 0.61 | | 0.15 | | 0.22 | | 0.04 | | 0.12 | | 0.11 | | 0.96 | | 0.49 | | 2.82 | | 4.75 | | 9.06 | |
| SD | | | 1.693 | | 13.258 | | 1.012 | | 0.332 | | 1.374 | | 0.543 | | 1.366 | | 2.383 | | 5.895 | | 14.651 | | 7.219 | |

| observation | geographical origin | year | amino acid (%) Met | SD | Hyp | SD | Phe | SD | Cys | SD | Gln | SD | Orn | SD | Lys | SD | His | SD | Tyr | SD | Trp | SD | Σ (μg/kg) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Amarante | 2000 | 0.07 | 0.005 | 3.17 | 0.253 | 1.53 | 0.017 | 1.10 | 0.042 | 0.55 | 0.040 | 0.23 | 0.006 | 2.37 | 0.063 | 21.96 | 1.371 | 0.21 | 0.011 | 3.83 | 0.163 | 526.2 |
| 2 | | 2001 | 0.09 | 0.004 | 18.90 | 0.444 | 0.76 | 0.026 | 0.54 | 0.021 | 1.81 | 0.060 | 0.11 | 0.008 | 0.41 | 0.019 | 3.15 | 0.184 | 0.04 | 0.004 | 0.34 | 0.011 | 1575.5 |
| 3 | | 2002 | 0.24 | 0.009 | 7.95 | 0.278 | 0.26 | 0.022 | 9.77 | 0.141 | 2.66 | 0.068 | 0.45 | 0.004 | 1.68 | 0.037 | 4.18 | 0.151 | 0.27 | 0.008 | 0.45 | 0.004 | 480.4 |
| 4 | Bairão | 2000 | 0.13 | 0.002 | 2.38 | 0.166 | 1.27 | 0.005 | 2.65 | 0.131 | 1.95 | 0.038 | 0.78 | 0.092 | 3.64 | 0.197 | 1.49 | 0.021 | 0.33 | 0.010 | 0.76 | 0.023 | 1044.6 |
| 5 | | 2001 | 0.06 | 0.001 | 6.58 | 0.162 | 0.02 | 0.001 | 1.91 | 0.036 | 0.68 | 0.008 | 0.13 | 0.001 | 1.63 | 0.037 | 2.61 | 0.119 | 0.02 | 0.001 | 0.05 | 0.001 | 1424.9 |
| 6 | | 2002 | 0.07 | 0.001 | 4.97 | 0.065 | 0.37 | 0.014 | 2.13 | 0.047 | 0.91 | 0.039 | 0.37 | 0.011 | 2.28 | 0.098 | 16.43 | 0.745 | 0.29 | 0.012 | 0.57 | 0.018 | 1355.7 |
| 7 | Bragança | 2000 | 0.17 | 0.010 | 19.49 | 0.393 | 0.28 | 0.016 | 2.27 | 0.087 | 3.31 | 0.219 | 0.15 | 0.011 | 2.03 | 0.081 | 2.51 | 0.048 | 0.04 | 0.001 | 1.77 | 0.085 | 1055.4 |
| 8 | | 2001 | 0.15 | 0.013 | 16.66 | 0.844 | 0.03 | 0.001 | 1.83 | 0.028 | 0.50 | 0.007 | 0.11 | 0.005 | 0.57 | 0.012 | 1.19 | 0.060 | 0.16 | 0.009 | 0.11 | 0.001 | 1236.7 |
| 9 | | 2002 | 0.34 | 0.016 | 9.58 | 0.470 | 0.34 | 0.008 | 0.66 | 0.008 | 0.12 | 0.002 | 0.09 | 0.002 | 0.68 | 0.006 | 10.84 | 0.093 | 0.08 | 0.001 | 1.42 | 0.015 | 1714.5 |
| 10 | Caminha | 2001 | 0.13 | 0.001 | 15.19 | 0.663 | 0.05 | 0.002 | 1.12 | 0.022 | 0.46 | 0.054 | 0.02 | 0.001 | 0.79 | 0.034 | 0.96 | 0.038 | 0.01 | 0.001 | 0.12 | 0.008 | 1587.3 |
| 11 | Covilhã | 2000 | 0.81 | 0.031 | 12.97 | 0.492 | 0.21 | 0.003 | 11.91 | 0.672 | 1.16 | 0.025 | 0.14 | 0.007 | 1.67 | 0.056 | 1.37 | 0.074 | 0.07 | 0.002 | 0.10 | 0.002 | 579.6 |
| 12 | | 2001 | 0.39 | 0.013 | 2.06 | 0.051 | 0.04 | 0.001 | 4.24 | 0.158 | 1.86 | 0.031 | 0.04 | 0.001 | 0.66 | 0.007 | 3.14 | 0.146 | 0.01 | 0.001 | 0.04 | 0.001 | 390.8 |
| 13 | | 2002 | 0.14 | 0.007 | 4.60 | 0.131 | 0.15 | 0.004 | 0.45 | 0.007 | 1.13 | 0.021 | 0.18 | 0.005 | 0.37 | 0.003 | 12.32 | 0.990 | 0.07 | 0.002 | 1.73 | 0.043 | 3113.9 |
| 14 | Custóias | 2001 | 0.02 | 0.002 | 30.26 | 1.597 | 0.09 | 0.001 | 0.67 | 0.031 | 0.62 | 0.029 | 0.01 | 0.001 | 0.21 | 0.007 | 0.64 | 0.024 | 0.07 | 0.002 | 0.14 | 0.001 | 3054.1 |
| 15 | | 2002 | 0.07 | 0.001 | 11.88 | 0.327 | 0.20 | 0.003 | 0.16 | 0.008 | 0.77 | 0.014 | 0.18 | 0.004 | 1.03 | 0.014 | 18.03 | 0.570 | 0.04 | 0.001 | 1.18 | 0.033 | 2259.0 |
| 16 | Pinhel | 2000 | 0.35 | 0.016 | 19.50 | 0.376 | 1.05 | 0.038 | 4.15 | 0.223 | 2.12 | 0.022 | 0.10 | 0.002 | 0.90 | 0.053 | 1.11 | 0.095 | 0.09 | 0.003 | 0.92 | 0.029 | 1356.6 |
| 17 | | 2001 | 0.01 | 0.001 | 4.70 | 0.194 | 0.10 | 0.005 | 1.69 | 0.105 | 0.71 | 0.056 | 0.16 | 0.011 | 0.43 | 0.011 | 2.93 | 0.161 | 0.02 | 0.002 | nd | | 758.8 |
| 18 | | 2002 | 0.50 | 0.007 | 5.93 | 0.252 | 0.34 | 0.010 | 2.71 | 0.068 | 1.01 | 0.008 | 0.02 | 0.001 | 1.04 | 0.032 | 6.67 | 0.280 | 0.13 | 0.001 | 0.72 | 0.026 | 946.9 |
| 19 | Vila Real | 2000 | 0.36 | 0.003 | 9.64 | 0.525 | 0.64 | 0.008 | 4.13 | 0.119 | 0.31 | 0.023 | 0.08 | 0.003 | 1.21 | 0.031 | 0.75 | 0.031 | 0.05 | 0.001 | 0.55 | 0.021 | 771.9 |
| 20 | | 2001 | 0.05 | 0.002 | 9.53 | 0.371 | 0.38 | 0.007 | 0.74 | 0.016 | 0.32 | 0.014 | 0.16 | 0.003 | 1.24 | 0.024 | 5.22 | 0.215 | 0.07 | 0.005 | 0.24 | 0.003 | 707.1 |
| 21 | | 2002 | 0.03 | 0.001 | 18.25 | 0.175 | 0.59 | 0.008 | 3.36 | 0.093 | 0.62 | 0.013 | 0.01 | 0.001 | 0.68 | 0.011 | 5.32 | 0.087 | 0.09 | 0.002 | 1.03 | 0.051 | 1918.3 |
| 22 | Viseu | 2000 | 0.34 | 0.024 | 16.85 | 0.834 | 0.19 | 0.005 | 5.24 | 0.268 | 2.60 | 0.052 | 0.42 | 0.003 | 1.99 | 0.082 | 2.05 | 0.043 | 0.06 | 0.003 | 0.59 | 0.026 | 345.9 |
| 23 | | 2001 | 0.37 | 0.003 | 15.36 | 0.919 | 0.06 | 0.001 | 5.28 | 0.181 | 1.74 | 0.004 | 0.02 | 0.002 | 0.62 | 0.022 | 1.23 | 0.095 | 0.10 | 0.005 | 0.46 | 0.044 | 868.1 |
| 24 | | 2002 | 0.09 | 0.001 | 12.98 | 0.319 | 0.80 | 0.005 | 0.73 | 0.025 | 0.56 | 0.022 | 0.13 | 0.004 | 0.64 | 0.026 | 12.77 | 0.432 | 0.08 | 0.002 | 1.31 | 0.035 | 1735.8 |
| mean | | | 0.21 | | 11.64 | | 0.41 | | 2.89 | | 1.19 | | 0.17 | | 1.20 | | 5.79 | | 0.10 | | 0.77 | | 1274.1 | |
| max | | | 0.81 | | 30.26 | | 1.53 | | 11.91 | | 3.31 | | 0.78 | | 3.64 | | 21.96 | | 0.33 | | 3.83 | | 3113.9 | |
| min | | | 0.01 | | 2.06 | | 0.02 | | 0.16 | | 0.12 | | 0.01 | | 0.21 | | 0.64 | | 0.01 | | nd | | 345.9 | |
| SD | | | 0.199 | | 6.994 | | 0.412 | | 2.905 | | 0.863 | | 0.177 | | 0.821 | | 6.184 | | 0.089 | | 0.844 | | 762.03 | |

[a] SD, standard deviation of three determinations; Σ, sum of the determined free amino acids; Ala, alanine; Gly, glycine; Val, valine; Leu, leucine; Ile, isoleucine; Pro, proline; Thr, threonine; Ser, serine; Glu, glutamic acid; Asn, asparagine; Asp, aspartic acid; nd, not detected; Σ, sum of the determined free amino acids; Met, methionine; Hyp, hydroxyproline; Phe, phenylalanine; Cys, cysteine; Gln, glutamine; Orn, ornithine; Lys, lysine; His, histidine; Tyr, tyrosine; Trp, tryptophan.

**Table 6.** Free Amino Acid Composition of Quince Peels[a]

| | | | amino acid (%) | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| observation | geographical origin | year | Ala | SD | Gly | SD | Val | SD | Leu | SD | Ile | SD | Pro | SD | Thr | SD | Ser | SD | Glu | SD | Asn | SD | Asp | SD |
| 25 | Amarante | 2000 | 2.40 | 0.082 | 2.20 | 0.055 | 2.21 | 0.072 | 0.81 | 0.014 | 2.49 | 0.114 | 0.94 | 0.041 | 1.18 | 0.047 | 2.39 | 0.228 | 8.38 | 0.293 | 22.56 | 2.022 | 12.56 | 0.418 |
| 26 | | 2001 | 2.06 | 0.099 | 2.69 | 0.056 | 0.08 | 0.002 | 0.11 | 0.002 | 0.47 | 0.010 | 0.37 | 0.006 | 2.46 | 0.048 | 1.03 | 0.041 | 19.27 | 0.947 | 36.22 | 0.492 | 15.57 | 0.320 |
| 27 | | 2002 | 1.62 | 0.033 | 0.46 | 0.020 | 0.22 | 0.035 | 0.13 | 0.003 | 0.91 | 0.048 | 0.62 | 0.018 | 2.07 | 0.070 | 10.18 | 0.058 | 47.03 | 1.346 | 1.14 | 0.075 | 9.42 | 0.231 |
| 28 | Baião | 2000 | 3.85 | 0.073 | 19.51 | 0.347 | 3.46 | 0.067 | 1.01 | 0.015 | 6.39 | 0.121 | 1.37 | 0.024 | 2.85 | 0.037 | 8.00 | 0.115 | 12.73 | 0.284 | 6.19 | 0.118 | 13.89 | 0.336 |
| 29 | | 2001 | 2.43 | 0.086 | 0.77 | 0.028 | 0.59 | 0.047 | 0.23 | 0.004 | 0.88 | 0.032 | 0.66 | 0.024 | 4.82 | 0.092 | 4.42 | 0.073 | 39.89 | 1.825 | 7.00 | 0.142 | 18.65 | 0.500 |
| 30 | | 2002 | 3.49 | 0.150 | 23.02 | 0.863 | 0.12 | 0.003 | 0.18 | 0.006 | 2.11 | 0.092 | 0.26 | 0.011 | 2.17 | 0.096 | 1.61 | 0.090 | 26.77 | 0.814 | 3.39 | 0.163 | 19.77 | 0.961 |
| 31 | Bragança | 2000 | 1.48 | 0.079 | 23.11 | 1.367 | 1.78 | 0.061 | 0.37 | 0.014 | 3.62 | 0.113 | 0.91 | 0.020 | 0.85 | 0.057 | 1.93 | 0.129 | 7.52 | 0.321 | 13.53 | 0.278 | 20.96 | 1.043 |
| 32 | | 2001 | 1.60 | 0.085 | 32.52 | 1.592 | 0.96 | 0.040 | 0.11 | 0.006 | 1.54 | 0.076 | 0.24 | 0.008 | 0.73 | 0.071 | 2.47 | 0.080 | 13.21 | 0.509 | 18.46 | 0.796 | 12.32 | 0.372 |
| 33 | | 2002 | 3.92 | 0.106 | 16.91 | 0.686 | 0.95 | 0.036 | 0.16 | 0.003 | 1.51 | 0.055 | 0.39 | 0.012 | 1.35 | 0.069 | 1.36 | 0.005 | 12.45 | 0.352 | 19.10 | 0.362 | 23.28 | 2.471 |
| 34 | Caminha | 2001 | 1.52 | 0.028 | 0.73 | 0.015 | 0.08 | 0.001 | 0.11 | 0.002 | 0.12 | 0.007 | 0.33 | 0.003 | 1.70 | 0.039 | 0.44 | 0.010 | 14.43 | 0.445 | 33.78 | 1.541 | 28.69 | 0.652 |
| 35 | Covilhã | 2000 | 1.54 | 0.043 | 44.98 | 2.660 | 1.77 | 0.062 | 0.21 | 0.009 | 5.02 | 0.111 | 0.49 | 0.009 | 1.28 | 0.072 | 3.02 | 0.183 | 3.20 | 0.098 | 5.77 | 0.091 | 14.83 | 0.807 |
| 36 | | 2001 | 1.11 | 0.067 | 5.46 | 0.277 | 0.48 | 0.016 | 0.19 | 0.003 | 0.56 | 0.034 | 0.37 | 0.007 | 1.42 | 0.063 | 0.65 | 0.003 | 10.83 | 0.360 | 36.84 | 0.795 | 10.43 | 0.070 |
| 37 | | 2002 | 0.95 | 0.018 | 7.63 | 0.158 | 0.82 | 0.020 | 0.11 | 0.003 | 0.61 | 0.013 | 0.20 | 0.003 | 0.19 | 0.008 | 2.92 | 0.053 | 14.01 | 0.698 | 24.52 | 0.802 | 14.53 | 0.236 |
| 38 | Custóias | 2001 | 1.99 | 0.080 | 0.26 | 0.007 | 1.88 | 0.100 | 0.20 | 0.005 | 0.49 | 0.023 | 2.35 | 0.125 | 4.09 | 0.110 | 1.58 | 0.049 | 15.97 | 0.835 | 31.75 | 0.798 | 18.69 | 0.717 |
| 39 | | 2002 | 1.64 | 0.022 | 5.60 | 0.178 | 0.57 | 0.016 | 0.06 | 0.002 | 0.26 | 0.007 | 0.35 | 0.008 | 0.21 | 0.007 | 3.75 | 0.110 | 15.08 | 0.439 | 24.56 | 0.474 | 17.48 | 0.378 |
| 40 | Pinhel | 2000 | 3.56 | 0.132 | 16.96 | 0.923 | 1.83 | 0.078 | 0.34 | 0.005 | 2.17 | 0.076 | 0.70 | 0.019 | 0.92 | 0.035 | 4.37 | 0.060 | 11.09 | 0.363 | 8.33 | 0.223 | 37.72 | 0.235 |
| 41 | | 2001 | 0.91 | 0.029 | 40.05 | 1.580 | 0.96 | 0.033 | 0.17 | 0.009 | 1.81 | 0.038 | 0.10 | 0.004 | 0.90 | 0.029 | 0.98 | 0.058 | 20.70 | 0.673 | 9.58 | 0.243 | 13.42 | 0.503 |
| 42 | | 2002 | 1.27 | 0.020 | 13.14 | 0.491 | 1.03 | 0.017 | 0.18 | 0.004 | 1.11 | 0.042 | 0.22 | 0.006 | 0.26 | 0.002 | 3.13 | 0.003 | 24.05 | 0.397 | 16.46 | 0.467 | 10.21 | 0.169 |
| 43 | Vila Real | 2000 | 7.14 | 0.079 | 19.41 | 1.452 | 1.26 | 0.053 | 0.26 | 0.006 | 4.37 | 0.124 | 1.85 | 0.083 | 2.83 | 0.121 | 5.50 | 0.302 | 7.28 | 0.032 | 19.92 | 0.713 | 11.99 | 0.488 |
| 44 | | 2001 | 2.14 | 0.006 | 16.69 | 0.732 | 0.82 | 0.024 | 0.14 | 0.007 | 0.94 | 0.022 | 0.42 | 0.012 | 1.05 | 0.056 | 2.03 | 0.040 | 23.47 | 0.883 | 17.63 | 0.135 | 14.78 | 0.045 |
| 45 | | 2002 | 1.28 | 0.056 | 13.13 | 0.594 | 0.59 | 0.001 | 0.12 | 0.007 | 0.75 | 0.021 | 0.39 | 0.012 | 0.40 | 0.011 | 2.29 | 0.057 | 24.21 | 0.795 | 13.40 | 0.455 | 17.49 | 0.799 |
| 46 | Viseu | 2000 | 3.28 | 0.103 | 23.49 | 0.519 | 2.40 | 0.062 | 0.22 | 0.011 | 2.44 | 0.059 | 1.31 | 0.036 | 0.47 | 0.019 | 3.03 | 0.128 | 3.42 | 0.082 | 17.45 | 0.621 | 23.85 | 1.038 |
| 47 | | 2001 | 0.96 | 0.037 | 0.73 | 0.024 | 0.69 | 0.031 | 0.11 | 0.003 | 0.30 | 0.011 | 0.25 | 0.009 | 1.66 | 0.156 | 0.99 | 0.013 | 14.57 | 0.503 | 35.55 | 1.034 | 19.52 | 0.330 |
| 48 | | 2002 | 3.08 | 0.061 | 1.02 | 0.036 | 0.86 | 0.035 | 0.17 | 0.006 | 0.34 | 0.042 | 0.55 | 0.007 | 1.51 | 0.045 | 3.27 | 0.123 | 42.77 | 0.933 | 4.21 | 0.013 | 18.38 | 0.956 |
| mean | | | 2.30 | | 13.77 | | 1.10 | | 0.23 | | 1.76 | | 0.65 | | 1.56 | | 2.97 | | 18.01 | | 17.81 | | 17.43 | |
| max | | | 7.14 | | 44.98 | | 3.46 | | 1.01 | | 6.89 | | 2.35 | | 4.82 | | 10.18 | | 47.03 | | 36.84 | | 37.72 | |
| min | | | 0.91 | | 0.26 | | 0.08 | | 0.06 | | 0.12 | | 0.10 | | 0.19 | | 0.44 | | 3.20 | | 1.14 | | 9.42 | |
| SD | | | 1.407 | | 12.919 | | 0.834 | | 0.223 | | 1.696 | | 0.559 | | 1.185 | | 2.296 | | 11.626 | | 11.131 | | 6.390 | |

| | | | amino acid (%) | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| observation | geographical origin | year | Met | SD | Hyp | SD | Phe | SD | Cys | SD | Gln | SD | Orn | SD | Lys | SD | His | SD | Tyr | SD | Trp | SD | Σ (μg/kg) |
| 25 | Amarante | 2000 | 0.35 | 0.010 | 4.82 | 0.308 | 1.78 | 0.040 | 4.92 | 0.164 | 3.56 | 0.158 | 0.15 | 0.013 | 3.71 | 0.153 | 13.86 | 0.586 | 0.08 | 0.006 | 8.65 | 0.164 | 616.3 |
| 26 | | 2001 | 0.08 | 0.008 | 11.58 | 0.607 | 0.61 | 0.007 | 1.98 | 0.086 | 2.04 | 0.042 | 0.17 | 0.002 | 0.86 | 0.025 | 1.82 | 0.001 | 0.18 | 0.001 | 0.35 | 0.004 | 793.6 |
| 27 | | 2002 | 0.34 | 0.008 | 3.93 | 0.070 | 0.18 | 0.008 | 13.75 | 0.406 | 3.27 | 0.079 | 0.11 | 0.009 | 1.41 | 0.033 | 2.80 | 0.189 | 0.15 | 0.004 | 0.26 | 0.004 | 755.9 |
| 28 | Baião | 2000 | 0.02 | 0.001 | 9.51 | 0.205 | 0.75 | 0.014 | 1.90 | 0.025 | 1.49 | 0.034 | 0.33 | 0.005 | 2.72 | 0.054 | 2.99 | 0.054 | 0.31 | 0.005 | 0.23 | 0.005 | 1819.9 |
| 29 | | 2001 | 0.48 | 0.014 | 5.60 | 0.098 | 0.24 | 0.013 | 8.07 | 0.301 | 1.73 | 0.071 | 0.11 | 0.003 | 1.39 | 0.047 | 1.63 | 0.022 | 0.19 | 0.008 | 0.17 | 0.007 | 461.4 |
| 30 | | 2002 | 0.15 | 0.012 | 2.40 | 0.123 | 0.29 | 0.011 | 6.30 | 0.179 | 2.54 | 0.055 | 0.02 | 0.001 | 0.56 | 0.018 | 4.67 | 0.030 | 0.02 | 0.001 | 0.18 | 0.005 | 1861.8 |
| 31 | Bragança | 2000 | 0.13 | 0.001 | 13.36 | 0.872 | 0.40 | 0.026 | 2.71 | 0.119 | 0.80 | 0.021 | 0.07 | 0.001 | 1.78 | 0.063 | 3.63 | 0.074 | 0.17 | 0.010 | 0.92 | 0.056 | 1529.9 |
| 32 | | 2001 | 0.18 | 0.008 | 4.54 | 0.466 | 0.06 | 0.001 | 1.64 | 0.045 | 0.52 | 0.037 | 0.40 | 0.014 | 1.14 | 0.041 | 7.66 | 0.356 | 0.20 | 0.005 | 0.06 | 0.004 | 454.1 |
| 33 | | 2002 | 1.28 | 0.016 | 5.06 | 0.036 | 0.19 | 0.011 | 6.00 | 0.002 | 3.36 | 0.177 | 0.04 | 0.001 | 0.37 | 0.004 | 1.32 | 0.095 | 0.16 | 0.012 | 0.84 | 0.024 | 1213.2 |
| 34 | Caminha | 2001 | 0.14 | 0.006 | 13.14 | 0.324 | 0.16 | 0.004 | 1.45 | 0.015 | 0.97 | 0.031 | 0.01 | 0.001 | 0.95 | 0.040 | 0.89 | 0.038 | 0.01 | 0.001 | 0.35 | 0.011 | 1289.6 |
| 35 | Covilhã | 2000 | 0.36 | 0.016 | 10.97 | 0.524 | 0.11 | 0.007 | 4.84 | 0.359 | 0.21 | 0.005 | 0.09 | 0.005 | 0.83 | 0.063 | 0.38 | 0.005 | 0.04 | 0.001 | 0.07 | 0.002 | 700.1 |
| 36 | | 2001 | 0.25 | 0.014 | 24.81 | 0.782 | 0.18 | 0.006 | 1.90 | 0.038 | 1.13 | 0.032 | 0.10 | 0.005 | 0.60 | 0.025 | 2.34 | 0.113 | 0.11 | 0.005 | 0.32 | 0.018 | 2334.6 |
| 37 | | 2002 | 0.37 | 0.013 | 2.26 | 0.029 | 0.12 | 0.005 | 0.45 | 0.010 | 0.66 | 0.014 | 0.11 | 0.005 | 0.83 | 0.009 | 27.71 | 0.750 | 0.08 | 0.002 | 0.94 | 0.019 | 1816.8 |
| 38 | Custóias | 2001 | 0.16 | 0.004 | 14.11 | 0.703 | 0.25 | 0.006 | 1.27 | 0.036 | 2.03 | 0.080 | 0.24 | 0.005 | 0.56 | 0.019 | 1.68 | 0.005 | 0.28 | 0.012 | 0.18 | 0.002 | 1753.6 |
| 39 | | 2002 | 0.16 | 0.016 | 7.06 | 0.237 | 0.13 | 0.006 | 0.33 | 0.004 | 0.82 | 0.041 | 0.12 | 0.005 | 1.95 | 0.038 | 19.25 | 0.409 | 0.08 | 0.003 | 0.54 | 0.011 | 1150.1 |
| 40 | Pinhel | 2000 | 0.03 | 0.004 | 3.78 | 0.073 | 0.48 | 0.019 | 1.92 | 0.084 | 0.20 | 0.013 | 0.23 | 0.004 | 0.95 | 0.032 | 3.95 | 0.104 | 0.05 | 0.001 | 0.42 | 0.004 | 874.1 |
| 41 | | 2001 | 0.03 | 0.002 | 1.11 | 0.049 | 0.02 | 0.001 | 1.62 | 0.058 | 0.20 | 0.002 | 0.26 | 0.007 | 0.70 | 0.040 | 5.85 | 0.141 | 0.18 | 0.017 | 0.45 | 0.043 | 696.4 |
| 42 | | 2002 | 0.61 | 0.001 | 2.70 | 0.098 | 0.14 | 0.006 | 0.33 | 0.001 | 0.42 | 0.002 | 0.07 | 0.001 | 1.10 | 0.004 | 22.55 | 0.531 | 0.05 | 0.001 | 0.96 | 0.021 | 930.4 |
| 43 | Vila Real | 2000 | 0.77 | 0.049 | 2.92 | 0.015 | 0.57 | 0.019 | 10.00 | 0.242 | 0.54 | 0.001 | 0.18 | 0.006 | 2.10 | 0.098 | 0.93 | 0.047 | 0.09 | 0.009 | 0.10 | 0.004 | 511.7 |
| 44 | | 2001 | 0.02 | 0.002 | 8.97 | 0.511 | 0.36 | 0.004 | 1.19 | 0.028 | 1.72 | 0.032 | 0.09 | 0.004 | 1.23 | 0.024 | 5.81 | 0.204 | 0.05 | 0.003 | 0.47 | 0.011 | 903.5 |
| 45 | | 2002 | 0.10 | 0.004 | 8.02 | 0.208 | 0.46 | 0.012 | 0.72 | 0.029 | 0.11 | 0.009 | 0.07 | 0.003 | 0.64 | 0.022 | 15.03 | 0.423 | 0.07 | 0.002 | 0.71 | 0.013 | 1484.3 |
| 46 | Viseu | 2000 | 0.14 | 0.012 | 7.77 | 0.224 | 0.11 | 0.004 | 1.21 | 0.016 | 1.91 | 0.055 | 0.75 | 0.015 | 1.01 | 0.014 | 5.01 | 0.177 | 0.10 | 0.004 | 0.64 | 0.035 | 1017.3 |
| 47 | | 2001 | 0.03 | 0.002 | 14.14 | 1.084 | 0.15 | 0.001 | 0.80 | 0.030 | 1.29 | 0.009 | 0.02 | 0.002 | 0.65 | 0.007 | 6.35 | 0.117 | 0.06 | 0.001 | 1.17 | 0.022 | 1368.8 |
| 48 | | 2002 | 0.34 | 0.015 | 5.08 | 0.027 | 0.46 | 0.008 | 9.02 | 0.362 | 3.03 | 0.114 | 0.10 | 0.001 | 1.41 | 0.054 | 3.26 | 0.991 | 0.18 | 0.008 | 0.45 | 0.015 | 771.2 |
| mean | | | 0.27 | | 7.82 | | 0.34 | | 3.49 | | 1.44 | | 0.16 | | 1.23 | | 6.72 | | 0.12 | | 0.81 | | 1129.5 |
| max | | | 1.28 | | 24.81 | | 1.78 | | 13.75 | | 3.56 | | 0.75 | | 3.71 | | 27.71 | | 0.31 | | 8.65 | | 2334.6 |
| min | | | 0.02 | | 1.11 | | 0.02 | | 0.33 | | 0.11 | | 0.01 | | 0.37 | | 0.38 | | 0.01 | | 0.06 | | 454.1 |
| SD | | | 0.289 | | 5.449 | | 0.361 | | 3.617 | | 1.087 | | 0.159 | | 0.772 | | 7.424 | | 0.079 | | 1.699 | | 518.05 |

[a] SD, standard deviation of three determinations; Σ, sum of the determined free amino acids; Ala, alanine; Gly, glycine; Val, valine; Leu, leucine; Ile, isoleucine; Pro, proline; Thr, threonine; Ser, serine; Glu, glutamic acid; Asn, asparagine; Asp, aspartic acid; Σ, sum of the determined free amino acids; Met, methionine; Hyp, hydroxyproline; Phe, phenylalanine; Cys, cysteine; Gln, glutamine; Orn, ornithine; Lys, lysine; His, histidine; Tyr, tyrosine; Trp, tryptophan.

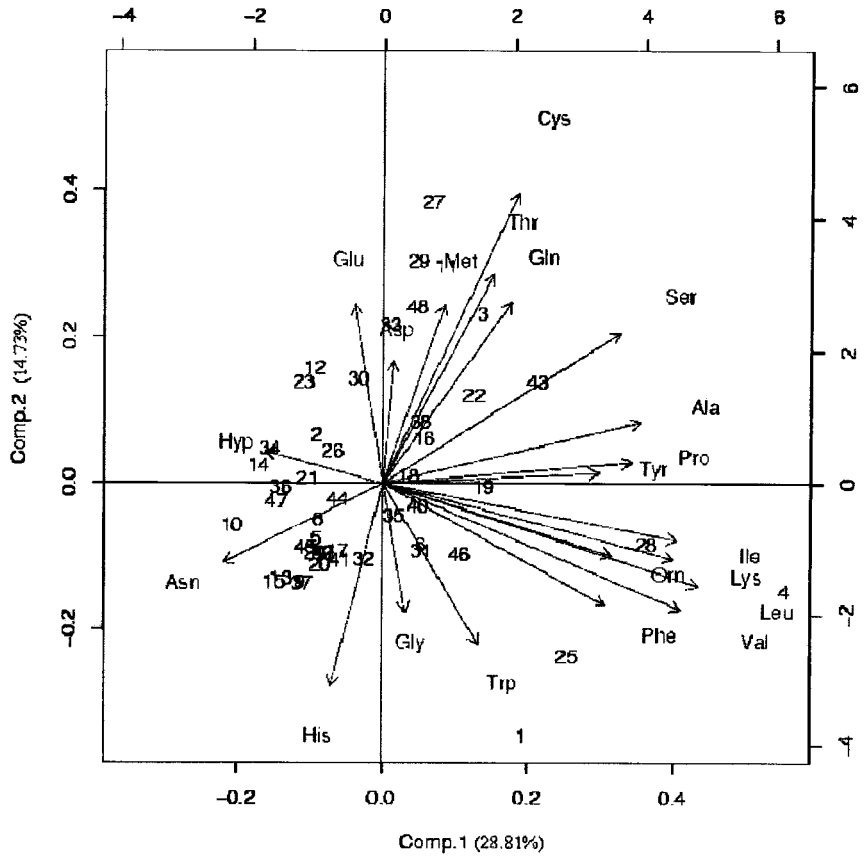


**Figure 3.** PCA of free amino acids in quince fruit, from 48 independent observations. Ala, alanine; Gly, glycine; Val, valine; Leu, leucine; Ile, isoleucine; Pro, proline; Thr, threonine; Ser, serine; Glu, glutamic acid; Asn, asparagine; Asp, aspartic acid; Met, methionine; Hyp, hydroxyproline; Phe, phenylalanine; Cys, cysteine; Gln, glutamine; Orn, ornithine; Lys, lysine; His, histidine; Tyr, tyrosine; Trp, tryptophan.

acid, and 2 kaempferol glycosides acylated with *p*-coumaric acid) (**Table 2**), as previously observed (*10*, *19*, *20*).

Generally, in quince pulp, the most abundant phenolic was 5-*O*-caffeoylquinic acid, whereas the major phenolic compound in quince peel was rutin. According to Silva et al. (*10*), in all studied cases, quince peel had a higher amount of phenolics than quince pulp. Absorption of UV light is a general feature of phenolic compounds (*26*). Some of them can be considered as filters that protect certain fragile cell structures (e.g., chloroplasts) from UV radiation. These filters consist mainly of flavonols and are located in the skins of fruits (*26*). In addition, because of their antioxidant properties, polyphenols can serve as protection against photooxidation caused by UV light (*26*). The antioxidant potential of quince pulp and peel methanolic extracts has already been reported (*20*). Peel methanolic extract exhibited greater antioxidant activity than the corresponding pulp extract, mainly due to the different qualitative and quantitative phenolic profile of these two parts of quince fruit.

The linear regression analysis (ANOVA full model) showed significant differences between the phenolic profiles of quince pulp and peel ($p < 0.001$). Significant differences were also found among the samples harvested in the three years, in terms of 3-*O*-caffeoylquinic acid ($p < 0.001$), 5-*O*-caffeoylquinic acid ($p < 0.05$) (only in pulps), and rutin ($p < 0.001$). Geographical origin did not influence significantly the phenolic composition of this fruit.

The differences between pulp and peel phenolic profiles were emphasized during PCA. Two main PCs accounted 81.29% of the total variability, PC1 (74.14%) and PC2 (7.15%) (**Figure 1**). PC1 is primarily responsible for the difference between the contents of caffeoylquinic acids (3-*O*-, 4-*O*-, and 5-*O*-caffeoylquinic acids and 3,5-*O*-dicaffeoylquinic acid) and flavonoids (quercetin 3-galactoside, rutin, kaempferol glycoside, kaempferol 3-glucoside, kaempferol 3-rutinoside, quercetin glycosides acylated with *p*-coumaric acid, and kaempferol glycosides acylated with *p*-coumaric acid). This characterizes the difference in the phenolic composition of pulp and peel. For example, quince pulp had an average content of 3-*O*-caffeoylquinic acid of 30.51 ± 10.058%, whereas peel had an average value of 11.77 ± 7.426%; peel had an average content of kaempferol-3-rutinoside of 3.95 ± 1.890%, whereas in pulp this flavonoid was absent (**Tables 1** and **2**). PC2 relates the content of 4-*O*-caffeoylquinic acid against the contents of 5-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids.

Generally, peel had a lower dispersion in terms of caffeoylquinic acids and flavonoids composition, making it possible to pool the data. However, the pulps had significant differences in the caffeoylquinic acids composition. Here, it is possible to observe three main groups: one rich in 4-*O*-caffeoylquinic acid and poor in 5-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids (observations 2, 10, and 24); another rich in 5-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids and poor in 4-*O*-caffeoylquinic acid (observations 1, 4, and 6); and another with average

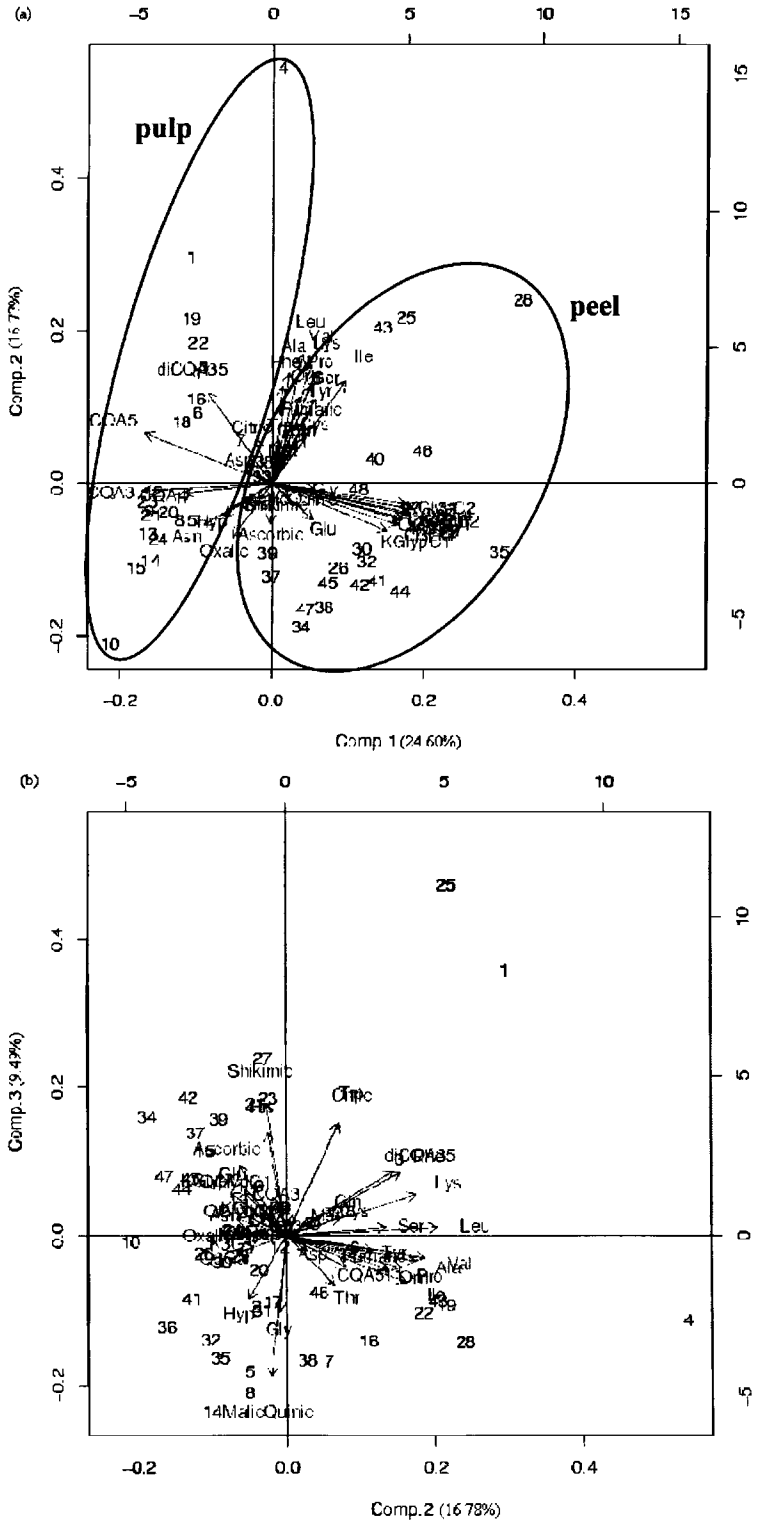


**Figure 4.** PCA of phenolics, organic acids, and free amino acids in quince fruit, from 48 independent observations: (**a**) PC1 versus PC2; (**b**) PC2 versus PC3. CQA3, 3-*O*-caffeoylquinic acid; CQA4, 4-*O*-caffeoylquinic acid; CQA5, 5-*O*-caffeoylquinic acid; diCQA35, 3,5-*O*-dicaffeoylquinic acid; Q3Gal, quercetin 3-galactoside; Q3Rut, rutin; K3Gly, kaempferol 3-glycoside; K3Glu, kaempferol 3-glucoside; K3Rut, kaempferol 3-rutinoside; QGly-pC1 and QGly-pC2, quercetin glycosides acylated with *p*-coumaric acid; KGly-pC1 and KGly-pC2, kaempferol glycosides acylated with *p*-coumaric acid; Ala, alanine; Gly, glycine; Val, valine; Leu, leucine; Ile, isoleucine; Pro, proline; Thr, threonine; Ser, serine; Glu, glutamic acid; Asn, asparagine; Asp, aspartic acid; Met, methionine; Hyp, hydroxyproline; Phe, phenylalanine; Cys, cysteine; Gln, glutamine; Orn, ornithine; Lys, lysine; His, histidine; Tyr, tyrosine; Trp, tryptophan.

composition (the rest of the observations), which may indicate the occurrence of caffeoylquinic acids isomerization in pulp matrix, once, according to Macheix et al. (*26*), transesterification of caffeoylquinic acids appears to be possible in fruit matrices.

From the food quality control point of view, it is very important to distinguish between quince pulp and peel, because Portuguese legislation (*1*) forbids the use of peel in the manufacture of quince jam.

**Organic Acids.** As previously reported (*11*), generally, the pulp and peel had similar profiles composed of seven identified organic acids: oxalic, citric, ascorbic, malic, quinic, shikimic, and fumaric acids (**Tables 3** and **4**). Quince fruit is characterized by large amounts of malic plus quinic acids, both in pulp and in peel, containing an average value of 96.45%, with maximum and minimum values of 99.46 and 90.46%, respectively. The ANOVA detected significant differences in the composition of quince fruits collected in the years 2000, 2001, and 2002, in terms of ascorbic acid ($p < 0.05$), shikimic acid ($p < 0.05$), fumaric acid ($p < 0.01$), and total organic acid content ($p < 0.001$), leading to the occurrence of a small decrease of organic acid total content for years 2000−2002. The part of the fruit and the geographical location did not influence significantly the organic acid composition of quince fruit.

Two PCs characterized the quince fruit organic acids composition (responsible for 61.61% of total variation). PC1 describes the domain of malic plus quinic acids on the quince fruit organic acid composition (37.54% of all variation). PC2 describes the orthogonality between oxalic plus ascorbic acids and citric acid in some quince fruits (24.07% of total variation). It is possible to observe that most samples presented large proportions of malic plus quinic acids, lowering the content of the other acids. **Figure 2** shows the high orthogonality between the oxalic plus ascorbic acids and citric acid. Some samples were very rich in terms of citric acid, with very low ascorbic and oxalic acids contents (observations 1 and 23), and others were rich in oxalic plus ascorbic acids but poor in citric acid (samples 15 and 44). It is also possible to observe some samples (3, 21, 27, 34, 39, and 42) balanced in terms of oxalic, ascorbic, and citric acids. In this case, in the PCA pulp and peel could not be distinguished.

**Free Amino Acids.** The quince fruit free amino acid profile was highly dispersed among the 21 constituents (**Tables 5** and **6** and **Figure 3**). Nevertheless, this fruit is richer in terms of asparagine (20.52%), aspartic acid (18.63%), glycine (12.32%), glutamic acid (11.66% for pulps and 18.01% for peels), hydroxyproline (11.64% for pulps and 7.82% for peels), and histidine (6.26%).

The ANOVA showed that some free amino acids contents vary significantly between harvesting year [Ala ($p < 0.05$), Val ($p < 0.001$), Leu ($p < 0.001$), Ile ($p < 0.001$), Pro ($p < 0.001$), Glu ($p < 0.001$), Phe ($p < 0.01$), Orn ($p < 0.05$), Lys ($p < 0.01$), and His ($p < 0.001$)] and geographical origin [Leu ($p < 0.05$), Lys ($p < 0.01$), and Tyr ($p < 0.05$)]. Generally, the free amino acids profiles are similar in pulp and peel. Nevertheless, the hydroxyproline content is significantly higher in pulp, whereas the glutamic acid content is significantly lower in this part of the fruit ($p < 0.05$).

The large dispersion in free amino acids composition led to a large number of PCs with significant variation ($n = 6$, >5% of the total variation). The first two PCs account for 43.54% of the total variability (28.81 and 14.73%, respectively) (**Figure 3**). PC1 represents the ratio of alanine, valine, leucine, isoleucine, proline, threonine, serine, phenylalanine, cysteine, glutamine, ornithine, lysine, and tyrosine against asparagine and hydroxyproline contents. PC2 describes the ratio of threonine, serine, glutamic acid, aspartic acid, methionine, cysteine, and glutamine against glycine, valine, leucine, asparagine, phenylalanine, ornithine, lysine, histidine, and tryptophan contents. **Figure 3** shows that all observations lie around the PC1 and PC2 center. The large variability of the free amino acids profile allows observations such as 4, 25, and 27, where the amounts of isoleucine, lysine, leucine, and valine (sample 4), phenylalanine and tryptophan (sample 25), and cysteine, threonine, methionine, and glutamic acid (sample 27) are unbalanced against the rest of the observations. In this case, in the PCA pulp and peel could not be distinguished.

**Global Analysis. Figure 4** presents the PCs of quince fruit composition (phenolics, organic acids, and free amino acids). Correlation analysis shows that there was no direct correlation among phenolics, organic acids, and free amino acids, so they are considered as independent observations. Three PCs explain 50.86% of the variability of all data: PC1 (24.60%), PC2 (16.78%), and PC3 (9.49%). PC1 emphasizes the differences in terms of phenolic compounds between pulp and peel. PC2 presents the differences between caffeoylquinic acids and flavonoids composition of pulp and peel, as well as the small differences in organic acids and free amino acids. PC3 describes the variation in terms of organic acids and the orthogonality existent between 3,5-*O*-dicaffeoylquinic acid and 4-*O*-caffeoylquinic acid.

**Conclusions.** After the analysis of several samples of quince pulp and peel of quince fruits from nine geographical locations in Portugal, harvested in three consecutive years (2000−2002), it can be concluded that phenolics determination is the most interesting with regard to the discrimination of these two parts of the fruit. The content of organic acids is very characteristic of quince fruit (both pulp and peel), being dominated by malic and quinic acids, the sum of which represents always >90% of the organic acids total content. Among the chemical parameters analyzed, the free amino acids profile is the most variable. Nevertheless, quince pulp is characterized by higher hydroxyproline and lower glutamic acid contents than peel. These data may be useful for the elaboration of a database for the detection of adulteration in quince products.

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# Principal component analysis as tool of characterization of quince (*Cydonia oblonga* Miller) jam

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**Abstract**

Fifty-one quince jams from several different brands, commercialised in three consecutive years, traditionally prepared and industrially manufactured, were studied. Principal component analysis (PCA) was performed, in order to assess the correlations between the different components of quince jam phenolics, organic acids and free amino acids. Phenolics determination was the most interesting. The differences between phenolic profiles of traditional and industrial quince jams were emphasised during PCA. Two main PC characterise the quince jam phenolic composition (54.4% of all variance): PC1 (37.4%) and PC2 (17.0%). The PC1 describes the differences between the contents of 3-*O*- and 5-*O*-caffeoylquinic acids and all flavonoids and the PC2 relates the contents of 4-*O*- and 5-*O*-caffeoylquinic acids against 3-*O*-caffeoylquinic and 3,5-dicaffeoylquinic acids. The results indicate that many industrial manufacturers usually use unpeeled fruits in the preparation of the jams. The PCA of phenolic compounds enabled clear discrimination between quince jams prepared with peeled and unpeeled fruits.
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*Keywords:* *Cydonia oblonga* Miller; Quince jam; Phenolic compounds; Organic acids; Free amino acids; Principal component analysis

## 1. Introduction

According to Portuguese Legislation (Decreto-Lei n° 97/84 de 28 de Março), quince jam is the food product of homogeneous and consistent mixture, obtained exclusively by boiling quince (*Cydonia oblonga* Miller) mesocarp with sugar. Because quince is a seasonal fruit, its homemade jam is prepared during September/October, by boiling a mixture composed only of sugar and quince pulp, normally in the proportion of 50:50. The industrially manufactured quince jam is prepared with quince puree, sugar and additives (preservatives such benzoic and sorbic acids, antioxidants such as ascorbic acid, acidity regulators such as citric and tartaric acids, etc).

In the past few years, the chemical constituents of quince fruit and its derivatives have been subject of study by our research group, (Andrade, Carvalho, Seabra, & Ferreira, 1998; Ferreres, Silva, Andrade, Seabra, & Ferreira, 2003; Silva, Andrade, Mendes et al., 2000; Silva, Andrade, Valentão et al., 2000; Silva, Andrade, Seabra, & Ferreira, 2001; Silva, Andrade, Mendes, Seabra, & Ferreira, 2002; Silva, Andrade, Ferreres et al., 2002; Silva et al., 2003; Silva, Casal et al., 2004; Silva, Andrade, Gonçalves et al., 2004; Silva, Andrade, Ferreres et al., 2005; Silva, 2005). Furthermore, past studies have included the evaluation of the antioxidant potential (Silva, Andrade, Valentão et al., 2004). Among the several chemical parameters studied, phenolic profile

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seemed to be the most useful in the evaluation quince product authenticity (Andrade et al., 1998; Silva, Andrade, Mendes et al., 2000; Silva, Andrade, Valentão et al., 2000; Silva et al., 2001; Silva, Andrade, Ferreres et al., 2002; Silva, Andrade, Gonçalves et al., 2004).

As the published results were only from organic acid and free amino acid composition of quince jams commercialised in 2000 (Silva, Andrade, Mendes et al., 2002; Silva, Casal et al., 2004), and considering the possibility of the influence of quince jam brand and the year of commercialisation on the chemical profile, the paper herein reports, for the first time, the organic acid and free amino acid composition of quince jams commercialised in 2001 and 2002. Principal component analysis (PCA) was applied to these data to assess the correlation between the different components of quince jam phenolics, organic acids and free amino acids. PCA was performed separately to each studied chemical parameter and to the global data.

## 2. Materials and methods

### 2.1. Samples

Forty-nine commercial quince jam samples were analysed and they included eight traditionally manufactured jams (observations 1–8) and 41 industrially manufactured jams (observations 11–51), randomly purchased on the Portuguese market in the years of 2000–2002 (Table 1). Additionally, a quince jam (observation 9) was prepared in the laboratory by boiling fresh quince pulps with sugar (in the proportion of 50:50), for approximately 90 min. Another quince jam (observation 10) was prepared by the same procedure, but using unpeeled fruits.

### 2.2. Standards

The standards were from Sigma (St. Louis, MO, USA) and from Extrasynthése (Genay, France). Methanol, formic and hydrochloric acids were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). Ethyl chloroformate (ECF) was from Aldrich (Steinheim, Germany) and pyridine from Fluka (Neu-Ulm, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

### 2.3. Solid-phase extraction columns

ISOLUTE C18 non end-capped (NEC) solid-phase extraction (SPE) columns (50 μm particle size, 60 Å porosity; 10 g sorbent mass/70 ml reservoir volume) were purchased from International Sorbent Technology Ltd (Mid Glamorgan, UK). The benzenesulfonic SCX Spe-ed SPE cartridges (200 mg; 3 ml) were obtained from Applied Separations (Allentown, USA).

Table 1
Samples characterization

| Observation | Brand | Year |
|---|---|---|
| 1 | A | 2000 |
| 2 | A | 2001 |
| 3 | A | 2002 |
| 4 | B | 2000 |
| 5 | B | 2001 |
| 6 | C | 2000 |
| 7 | C | 2001 |
| 8 | D | 2000 |
| 9 | Homemade 1 | 2002 |
| 10 | Homemade 2 | 2002 |
| 11 | E | 2000 |
| 12 | E | 2001 |
| 13 | E | 2002 |
| 14 | F | 2000 |
| 15 | F | 2001 |
| 16 | F | 2002 |
| 17 | G | 2000 |
| 18 | G | 2001 |
| 19 | G | 2002 |
| 20 | H | 2000 |
| 21 | H | 2001 |
| 22 | I | 2000 |
| 23 | I | 2001 |
| 24 | I | 2002 |
| 25 | J | 2000 |
| 26 | J | 2001 |
| 27 | J | 2002 |
| 28 | K | 2000 |
| 29 | K | 2001 |
| 30 | K | 2002 |
| 31 | L | 2000 |
| 32 | M | 2000 |
| 33 | M | 2001 |
| 34 | M | 2002 |
| 35 | N | 2000 |
| 36 | N | 2001 |
| 37 | N | 2002 |
| 38 | O | 2000 |
| 39 | O | 2001 |
| 40 | O | 2002 |
| 41 | P | 2000 |
| 42 | P | 2001 |
| 43 | P | 2002 |
| 44 | Q | 2000 |
| 45 | Q | 2001 |
| 46 | Q | 2002 |
| 47 | R | 2000 |
| 48 | R | 2001 |
| 49 | R | 2002 |
| 50 | S | 2000 |
| 51 | T | 2000 |

### 2.4. Extraction and HPLC analysis of phenolic compounds

The extraction of phenolics was achieved as previously reported (Silva et al., 2001; Silva, Andrade, Ferreres et al., 2002) and included a C18 NEC SPE cleaning step.

The extracts were analysed on an analytical HPLC unit (Gilson), using a Spherisorb ODS2 (25.0 × 0.46 cm; 5 μm, particle size) column (Silva et al., 2001; Silva, Andrade, Ferreres et al., 2002). Detection was achieved with a Gilson DAD.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3- and 4-*O*-caffeoylquinic, and 3,5-dicaffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid. Kaempferol glycoside and kaempferol glycosides acylated with *p*-coumaric acid were quantified as kaempferol 3-glucoside. Quercetin glycosides acylated with *p*-coumaric acid were quantified as quercetin 3-galactoside. The other compounds were quantified as themselves.

### 2.5. Extraction and HPLC analysis of organic acids

The sample preparation was simple, involving only extraction with methanol (40 °C) and filtration through a C18 NEC SPE cartridge, as reported by Silva, Andrade, Mendes et al. (2002).

The separation was carried out as previously reported (Silva, Andrade, Mendes et al., 2002) with an analytical HPLC unit (Gilson), using an ion exclusion Nucleogel® Ion 300 OA (300 × 7.7 mm) column. 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. Malic and quinic acids were quantified together and as malic acid. The other acids were quantified as themselves.

### 2.6. Extraction and GC of free amino acids

According to Silva et al. (2003), the extraction of L-amino acids was simple, including a SCX SPE purification step. The derivatisation of L-amino acids was carried out as reported previously (Silva et al., 2003). The extracts were analysed on a Chrompack CP 9001 instrument (Chrompack, Middelburg, The Netherlands) equipped with a flame ionisation detector (FID), and an automatic liquid sampler (CP-9050, Chrompack) (Silva et al., 2003).

The amino acids were identified by their retention times and chromatographic comparison with authentic standards. Quantification was based on the internal standard method using L-*p*-chlorophenylalanine.

### 2.7. Statistical analysis

All statistical analyses involving the experimental data were performed using R 1.9.0 for Linux (R-Project, 2004).

A multifactor ANOVA (without replication) was performed to evaluate the effects of the studied factors – quince jam brand, jam type (traditional or industrial) and the year of commercialisation (2000–2002) – on phenolics, organic acids and free amino acids.

The multifactor linear regression model was analysed for residuals normality and skewness to access the validity of the ANOVA analysis. Despicable factor effects were removed from the full linear model to improve the accuracy of the analysis. The ANOVA tables and factor probabilities and their combinations were obtained. The Tukey multicomparison test was used to perform pair-wise comparisons among factor levels means (Neter, Kutner, Natchtsheim, & Wasserman, 1996a).

The Pearson correlation coefficients between phenolics, organic acids and free amino acids were calculated to obtain possible correlation between the different quince jam constituents (Neter, Kutner, Natchtsheim, & Wasserman, 1996b).

#### 2.7.1. Principal component analysis

PCA was performed to access the correlations between the different components of quince jam phenolics, organic acids and free amino acids. PCA was performed separately to each studied chemical parameter (phenolic, organic acid and free amino acid profiles), but also to the global data.

Principal components (PC) were analysed for their variance percentage and component coefficients, to access their significance. The Gabriel plot (biplot), using optimal-scaling was performed to gain greater insight of relationships between quince jam components, aiming to interpret the different groups of data (Krazanowski, 1998).

## 3. Results and discussion

### 3.1. Experimental design

Quince jams were analysed in terms of phenolics, organic acids and free amino acids. The analysis comprised results from 51 samples, 10 traditionally prepared and 41 industrially manufactured, throughout the commercialisation years of 2000–2002 (Table 1). Although the full factorial design (Box, Hunter, & Hunter, 1978) was initially planned, it was not possible to obtain quince jam samples for every commercialisation year, so the analysis was carried out with the partial factorial design without replication (Montegomery, 1991a, 1991b).

### 3.2. Phenolic compounds

All quince jams presented a chemical profile composed by at least six identified phenolic compounds: 3-*O*-, 4-*O*- and 5-*O*-caffeoylquinic acids, 3,5-dicaffeoylquinic acid,

Table 2
Phenolic composition of quince jams

| | Phenolic compound (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3-CQA | 4-CQA | 5-CQA | 3,5-diCQA | Q-3-Gal | Q-3-Rut | K-3-Gly |
| *Traditional type quince jams* | | | | | | | |
| Mean | 32.8 | 12.1 | 44.4 | 1.97 | 1.04 | 6.51 | 0.11 |
| Maximum | 42.3 | 33.9 | 54.2 | 5.57 | 3.14 | 15.7 | 0.39 |
| Minimum | 11.9 | 5.63 | 31.6 | 0.20 | tr | 1.40 | nd |
| SD | 11.0 | 8.75 | 7.76 | 1.55 | 1.25 | 5.11 | 0.18 |
| | K-3-Glu | K-3-Rut | Q-Gly-pC1 | Q-Gly-pC2 | K-Gly-pC1 | K-Gly-pC2 | ∑ (mg/kg) |
| Mean | 0.14 | 0.34 | 0.14 | 0.10 | 0.07 | 0.21 | 176.3 |
| Maximum | 0.47 | 1.50 | 0.54 | 0.39 | 0.39 | 1.07 | 275.7 |
| Minimum | nd | nd | nd | nd | nd | nd | 112.2 |
| SD | 0.19 | 0.57 | 0.23 | 0.15 | 0.15 | 0.35 | 66.5 |
| | 3-CQA | 4-CQA | 5-CQA | 3,5-diCQA | Q-3-Gal | Q-3-Rut | K-3-Gly |
| *Industrial type quince jams* | | | | | | | |
| Mean | 26.3 | 9.25 | 42.7 | 1.83 | 3.05 | 12.9 | 0.92 |
| Maximum | 35.2 | 19.6 | 52.9 | 3.55 | 5.11 | 21.1 | 3.00 |
| Minimum | 8.43 | 4.46 | 34.4 | 0.65 | tr | 5.76 | Nd |
| SD | 4.99 | 3.97 | 4.36 | 0.65 | 1.35 | 3.23 | 0.52 |
| | K-3-Glu | K-3-Rut | Q-Gly-pC1 | Q-Gly-pC2 | K-Gly-pC1 | K-Gly-pC2 | ∑ (mg/kg) |
| Mean | 0.65 | 1.37 | 0.39 | 0.15 | 0.09 | 0.37 | 238.9 |
| Maximum | 1.59 | 3.09 | 1.38 | 0.69 | 0.42 | 1.05 | 417.3 |
| Minimum | nd | nd | nd | nd | nd | nd | 103.0 |
| SD | 0.33 | 0.56 | 0.26 | 0.13 | 0.12 | 0.19 | 81.4 |

SD, standard deviation; tr traces; nd, not detected; ∑, sum of the determined phenolic compounds; 3-CQA, 3-*O*-caffeoylquinic acid; 4-CQA, 4-*O*-caffeoylquinic acid; 5-CQA, 5-*O*-caffeoylquinic acid; 3,5-CQA, 3,5-dicaffeoylquinic acid; Q-3-Gal, quercetin 3-galactoside; Q-3-Rut, rutin, K-3-Gly - kaempferol 3-glycoside; K-3-Glu, kaempferol 3-glucoside; K-3-Rut, kaempferol 3-rutinoside; Q-Gly-pC1 and Q-Gly-pC2, quercetin glycosides acylated with *p*-coumaric acid; K-Gly-pC1 and K-Gly-pC2, kaempferol glycosides acylated with *p*-coumaric acid.

quercetin 3-galactoside and rutin (Table 2). Although, several samples, especially the industrially manufactured ones, also contained kaempferol 3-glucoside, kaempferol 3-rutinoside, and five not totally identified compounds (one kaempferol glycoside, two quercetin glycosides acylated with *p*-coumaric acid and two kaempferol glycosides acylated with *p*-coumaric acid) (Table 2). The presence of these compounds indicates adulteration of the jams by using unpeeled fruits (Silva, Andrade, Ferreres et al., 2002). Generally, the most abundant phenolic was 5-*O*-caffeoylquinic acid (average value of 43.0% minimum and maximum values of 31.6%, and 54.2% respectively).

The multifactor ANOVA analysis described very significant differences between the traditional and industrial quince jams phenolic profile (Table 2 and Fig. 1). It is possible to observe that traditional quince jams were richer in 3-*O*-caffeoylquinic acid ($p < 0$ 01). Industrial quince jams were, generally, richer in terms of flavonoids. Data show that industrial quince jams had higher content than the traditional ones in terms of: quercetin 3-galactoside ($p < 0.001$), rutin ($p < 0.05$), kaempferol glycoside ($p < 0.001$), kaempferol 3-glucoside ($p < 0.01$), kaempferol 3-rutinoside ($p < 0.01$) and quercetin glycosides acylated with *p*-coumaric acid ($p < 0.05$) (Fig. 1). The higher content of flavonoids in industrial quince jams indicates the presence of a higher proportion of quince peel in these jams. Some phenolics content also varied significantly according to the quince jam brand: 4-*O*-caffeoylquinic acid ($p < 0.01$), quercetin 3-galactoside ($p < 0.05$), rutin ($p < 0.001$), kaempferol glycoside ($p < 0.01$), kaempferol 3-glucoside ($p < 0.01$), kaempferol 3-rutinoside ($p < 0.001$) and total content ($p < 0.01$). Other phenolics varied significantly according to the year of commercialisation: quercetin 3-galactoside ($p < 0.01$), rutin ($p < 0.01$) and one of the kaempferol glycosides acylated with *p*-coumaric acid (KGly*p*C2) ($p < 0.05$).

The differences between traditional and industrial quince jams phenolic profiles were emphasised during PCA. Two main PC characterise to the quince jam phenolic composition (54.4% of all variance): PC1 (37.4%) and PC2 (17.0%) (Fig. 2). The PC1 describes the differences between the contents of 3-*O*- and 5-*O*-caffeoylquinic acids and all flavonoids (quercetin 3-galactoside, rutin, kaempferol glycoside, kaempferol 3-glucoside, kaempferol 3-rutinoside, quercetin glycosides acylated with *p*-coumaric acid and kaempferol glycosides acylated with *p*-coumaric acid). The PC2 relates the contents of 4-*O*- and 5-*O*-caffeoylquinic acids against 3-*O*-caffeoylquinic and 3,5-dicaffeoylquinic acids. It is possible to observe, in Fig. 2, a very distinct difference between traditional and industrial quince jams.

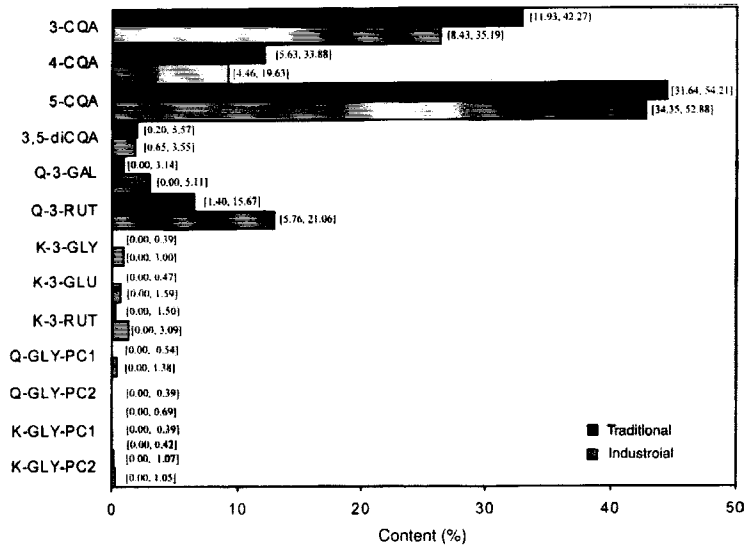


Fig. 1. Quince jam phenolic profile (minimum and maximum values of each compound are presented in brackets). 3-CQA, 3-*O*-caffeoylquinic acid; 4-CQA, 4-*O*-caffeoylquinic acid; 5-CQA, 5-*O*-caffeoylquinic acid; 3,5-diCQA, 3,5-dicaffeoylquinic acid; Q-3-GAL, quercetin 3-galactoside; Q-3-RUT, rutin; K-3-GLY, kaempferol 3-glycoside; K-3-GLU, kaempferol 3-glucoside; K-3-RUT, kaempferol 3-rutinoside; Q-GLY-PC1 and Q-GLY-PC2, quercetin glycosides acylated with *p*-coumaric acid; K-GLY-PC1 and K-GLY-PC2, kaempferol glycosides acylated with *p*-coumaric acid.

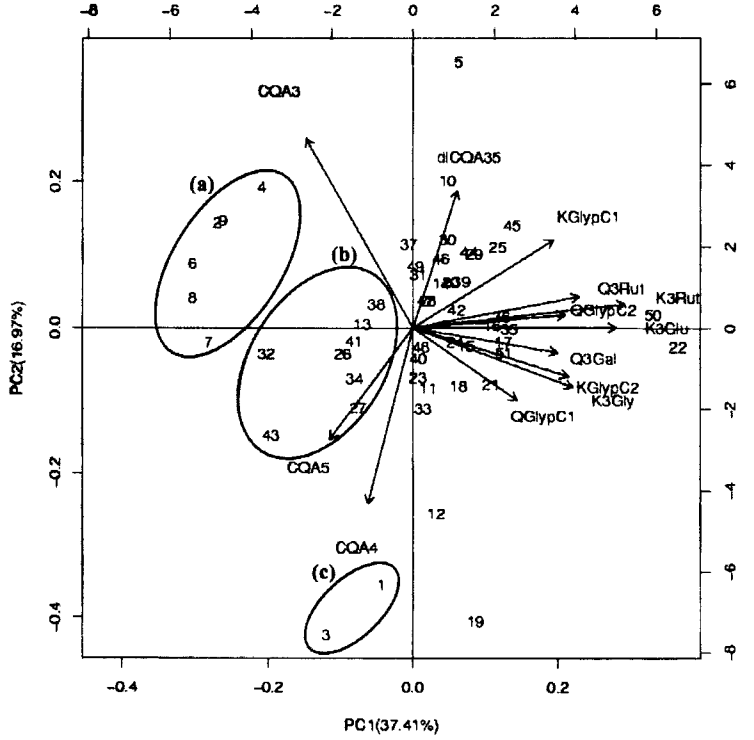


Fig. 2. Principal component analysis of phenolic compounds in quince jam, from 51 independent observations. CQA3, 3-*O*-caffeoylquinic acid; CQA4, 4-*O*-caffeoylquinic acid; CQA5, 5-*O*-caffeoylquinic acid; diCQA35, 3,5-dicaffeoylquinic acid; Q3Gal, quercetin 3-galactoside; Q3Rut, rutin; K3Gly, kaempferol 3-glycoside; K3Glu, kaempferol 3-glucoside; K3Rut, kaempferol 3-rutinoside; QGlypCl and QGlypC2, quercetin glycosides acylated with *p*-coumaric acid; KGlypCl and KGlypC2, kaempferol glycosides acylated with *p*-coumaric acid.

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Almost all industrial type jams are localised in the positive axis of PC1, indicating that, according to Portuguese Legislation (Decreto-Lei n° 97/84 de 28 Março), most of the samples were adulterated with quince peel. However, a small group of industrial type jams were prepared with peeled fruits or with a lower proportion of peel (Fig. 2 - group b). Traditional quince jams were, generally, characterised by higher amount of caffeoylquinic acids. It is possible to distinguish two different groups of traditional type jams (Fig. 2): group a – higher content in terms of 3-*O*-caffeoylquinic and 3,5-dicaffeoylquinic acids and group c – higher content of 4-*O*-caffeoylquinic acid. Previous studies on quince fruit composition have revealed that the pulp composition in terms of 4-*O*-caffeoylquinic and 3,5-dicaffeoylquinic acids is antagonistic (Silva, 2005). Generally, pulps with high 4-*O*-caffeoylquinic acid content exhibited low 3,5-dicaffeoylquinic acid and vice-versa (Silva, 2005). Under these circumstances, the difference in composition in terms of 4-*O*-caffeoylquinic acid and 3,5-dicaffeoylquinic acid was expected.

Table 3
Organic acid composition of quince jams

| | Organic acid (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Oxalic acid | Citric acid | Ascorbic acid | Malic and quinic acids | Shikimic acid | Fumaric acid | ∑ (mg/kg) |
| *Traditional type quince jams* | | | | | | | |
| Mean | 0.03 | 0.57 | 3.88 | 95.3 | 0.18 | 0.01 | 4518 |
| Maximum | 0.13 | 1.69 | 15.7 | 98.4 | 0.33 | 0.03 | 5894 |
| Minimum | nd | tr | 0.75 | 84.1 | 0.09 | nd | 2514 |
| SD | 0.04 | 0.60 | 4.72 | 4.66 | 0.09 | 0.01 | 1235 |
| *Industrial type quince jams* | | | | | | | |
| Mean | 0.06 | 17.4 | 2.16 | 80.2 | 0.15 | 0.01 | 7614 |
| Maximum | 1.25 | 58.9 | 13.0 | 97.9 | 0.69 | 0.04 | 12177 |
| Minimum | nd | 1.12 | tr | 32.7 | 0.02 | nd | 2988 |
| SD | 0.20 | 14.3 | 2.34 | 15.1 | 0.11 | 0.01 | 2629 |

SD, standard deviation; nd, not detected; tr, traces; ∑, sum of the determined organic acids.

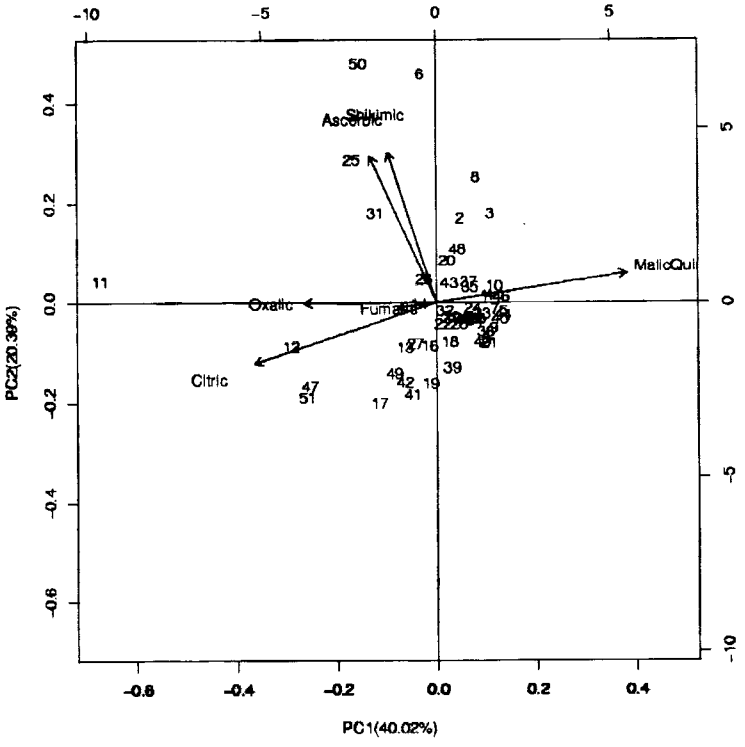


Fig. 3. Principal component analysis of organic acids in quince jam, from 51 independent observations.

One of the traditional jams (observation 10), prepared in the laboratory, by using unpeeled fruits revealed, as expected, a high content of flavonoids, which indicates the use of quince peel during manufacture.

### 3.3. Organic acids

As previously reported (Silva, Andrade, Mendes et al., 2002), generally, quince jams presented a similar profile composed of seven identified organic acids: oxalic, citric, ascorbic, malic, quinic, shikimic and fumaric acids (Table 3). Usually, quince jam is characterised by large amounts of malic plus quinic acids, containing an average value of 83.2% (with minimum and maximum values of 32.7% and 98.4%, respectively). Generally, citric and ascorbic acids were presented in considerable amounts, with average values of 14.1 and 2.50, respectively.

The multifactor ANOVA analysis described significant differences between the organic acid composition of traditional and industrial quince jams (Table 3), in terms of: citric acid ($p < 0.01$, higher in industrial type quince jams) and malic plus quinic acids of ($p < 0.01$, higher in traditional type quince jams). The contents of some organic acids also varied significantly according to the commercialisation year: ascorbic acid ($p < 0.05$), malic plus quinic acids ($p < 0.05$), fumaric acid ($p < 0.05$) and the total content ($p < 0.01$). Other organic acids varied significantly with quince jam brand: citric acid ($p < 0.05$), ascorbic acid ($p < 0.05$) and the total content ($p < 0.001$).

The organic acid profile can be described by two PC (Fig. 3), which are responsible to 60.4% of the variance. PC1 (40.0% of the total variation) expresses mainly the ratio malic plus quinic acids against the rest of the organic acids. PC2 (20.4% of the total variation) expresses mainly the citric acid content when compared to the rest of the acids. It is possible to observe, in Fig. 3, that most samples had high malic plus quinic acids ratios. However, samples 12, 47 and 51 exhibited high contents of citric acid and samples 6, 25, 31 and 50 had high ascorbic acid contents. This can be explained by the fact that, frequently, citric and ascorbic acids are added to industrially produced quince jam, as acidity regulator and antioxidant, respectively.

### 3.4. Free amino acids

Quince jams were richer in terms of aspartic acid (32.1%) and asparagine (30.0%), had medium values of glycine (8.01%), hydroxyproline (6.86%), threonine (3.93%), alanine (3.31%), glutamic acid (3.28%) and cysteine (3.11%), and very small proportions of the other free amino acids (Table 4).

Table 4
Free amino acid composition of quince jams

| Amino acid (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Traditional type quince jams* | | | | | | | | |
| | Ala | Gly | Val | Leu | Ile | Pro | Thr | Ser |
| Mean | 2.36 | 4.12 | 0.74 | 0.22 | 0.47 | 0.51 | 3.77 | 1.64 |
| Maximum | 4.37 | 12.16 | 2.83 | 0.52 | 0.94 | 1.12 | 11.2 | 3.04 |
| Minimum | 0.90 | 0.73 | 0.26 | 0.07 | 0.04 | 0.18 | 0.15 | 0.09 |
| SD | 1.25 | 4.08 | 0.76 | 0.13 | 0.30 | 0.28 | 3.17 | 0.92 |
| | Glu | Asn | Asp | Met | Hyp | Phe | Cys | Gln |
| Mean | 2.48 | 29.9 | 40.45 | 0.08 | 8.12 | 0.26 | 2.83 | 0.42 |
| Maximum | 7.61 | 64.9 | 68.24 | 0.29 | 30.0 | 0.49 | 6.28 | 0.63 |
| Minimum | 0.06 | 9.01 | 17.13 | 0.01 | 0.42 | 0.07 | 0.36 | 0.11 |
| SD | 2.43 | 18.1 | 19.48 | 0.09 | 8.86 | 0.15 | 2.22 | 0.21 |
| | Orn | Lys | His | Tyr | Trp | $\sum$ (μg/kg) | | |
| Mean | 0.17 | 0.56 | 0.68 | 0.15 | 0.07 | 993 | | |
| Maximum | 0.51 | 1.22 | 1.90 | 0.30 | 0.21 | 2401 | | |
| Minimum | 0.01 | 0.13 | 0.04 | 0.02 | 0.01 | 333 | | |
| SD | 0.18 | 0.41 | 0.62 | 0.11 | 0.09 | 601 | | |
| *Industrial type quince jams* | | | | | | | | |
| | Ala | Gly | Val | Leu | Ile | Pro | Thr | Ser |
| Mean | 3.55 | 8.96 | 0.91 | 0.34 | 1.43 | 0.54 | 3.97 | 2.18 |
| Maximum | 26.5 | 25.0 | 3.29 | 1.16 | 10.2 | 1.04 | 8.81 | 6.60 |
| Minimum | 0.03 | 0.23 | 0.21 | 0.11 | 0.34 | 0.19 | 0.79 | 0.62 |
| SD | 5.03 | 6.48 | 0.54 | 0.23 | 1.68 | 0.23 | 2.14 | 1.39 |
| | Glu | Asn | Asp | Met | Hyp | Phe | Cys | Gln |
| Mean | 3.47 | 30.0 | 30.1 | 0.06 | 6.55 | 0.38 | 3.18 | 1.11 |
| Maximum | 11.0 | 62.7 | 51.1 | 0.26 | 30.0 | 2.16 | 12.4 | 2.91 |
| Minimum | 0.87 | 4.51 | 8.60 | 0.01 | 0.69 | 0.02 | 0.07 | 0.04 |
| SD | 2.08 | 15.6 | 13.0 | 0.05 | 7.46 | 0.40 | 2.58 | 0.66 |
| | Orn | Lys | His | Tyr | Trp | $\sum$ (μg/kg) | | |
| Mean | 0.20 | 1.31 | 1.44 | 0.16 | 0.19 | 698 | | |
| Maximum | 1.19 | 4.09 | 8.73 | 0.73 | 1.48 | 1337 | | |
| Minimum | 0.02 | 0.25 | 0.14 | 0.02 | 0.01 | 309 | | |
| SD | 0.23 | 0.83 | 1.50 | 0.16 | 0.25 | 222 | | |

SD, standard deviation; $\sum$, sum of the determined free amino acids; ala, alanine; gly, glycine; val, valine; leu, leucine; ile, isoleucine; pro, proline; thr, threonine; ser, serine; glu, glutamic acid; asn, asparagine; asp, aspartic acid; met, methionine; hyp, hydroxyproline; phe, phenylalanine; cys, cysteine; gln, glutamine; orn, ornithine; lys, lysine; his, histidine; tyr, tyrosine; trp, tryptophan.

The multifactor ANOVA showed differences in the amino acids composition of traditional and industrial quince jams in terms of: glutamic acid ($p < 0.05$, higher in industrial quince jams), aspartic acid ($p < 0.05$, higher in traditional quince jams), glutamine ($p < 0.05$, higher in industrial quince jams), lysine ($p < 0.01$, higher in industrial quince jams), and total content ($p < 0.01$, higher in traditional quince jams). The content of some free amino acids also varied significantly according to the year of commercialisation: valine ($p < 0.01$), threonine ($p < 0.001$) and glutamic acid ($p < 0.001$). Glutamine content also varied significantly with the quince jam brand.

Two PC describe 38.5% of the total variance: PC1 (24.2%) relates the content of aspartic acid, hydroxyproline and asparagine with the rest of the free amino acids; PC2 (14.3%) relates the proportion of threonine, serine, proline, cysteine, glutamine and aspartic acid against histidine, tryptophan, phenylalanine, hydroxyproline and asparagine. In Fig. 4 (biplot of PC1 vs. PC2), it is possible to observe that most quince jams were rich in terms of

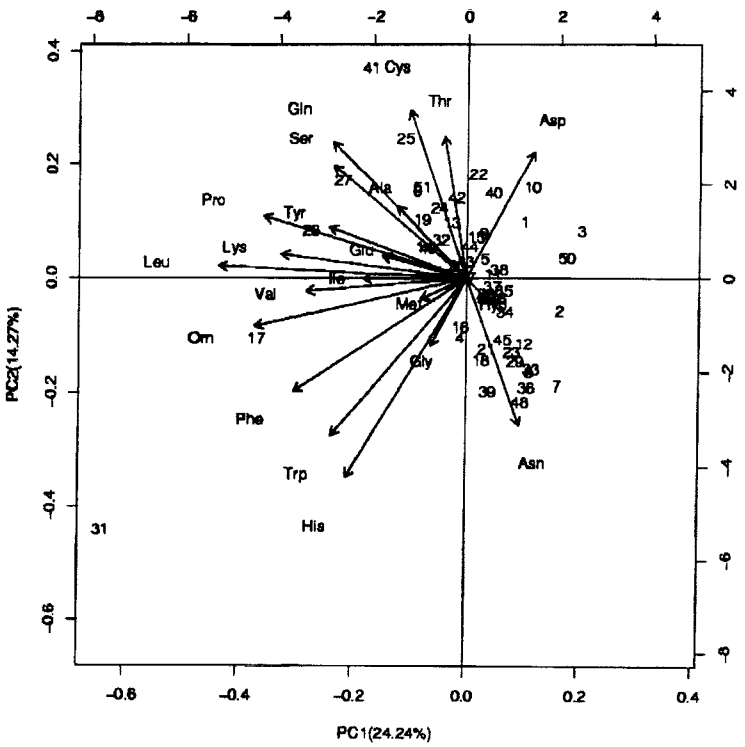


Fig. 4. Principal component analysis of free amino acids in quince jam, from 51 independent observations. Ala, alanine; gly, glycine; val, valine; leu, leucine; ile, isoleucine; pro, proline; thr, threonine; ser, serine; glu, glutamic acid; asn, asparagine; asp, aspartic acid; met, methionine; hyp, hydroxyproline; phe, phenylalanine; cys, cysteine; gln, glutamine; orn, ornithine; lys, lysine; his, histidine; tyr, tyrosine; trp, tryptophan.

aspartic acid and asparagine and the content in terms of the other amino acids is more fluctuating. Quince fruit and quince jam levels of asparagine and aspartic acid exhibited an inverse correlation. Quince fruit composition is higher in asparagine than in aspartic acid, but quince jam has higher aspartic acid than asparagine content. This can probably be explained by the fact that asparagine can be converted into aspartic acid and/or due to hydrolysis of proteins, peptides or other compounds with amino acids in their constitution, which can occur during thermal processing (in acid medium).

### 3.5. Global analysis

The PCA of all the data reveals that 26.9% of the variation sources can be described by two PCs: PC1 (14.8%) and PC2 (12.2%). Such small percentage of total variance does not suggest substantial differences between quince jams when all the studied parameters are compared. The correlation analysis between quince jam phenolics, organic acids and free amino acids contents leads to the conclusion that no direct correlation exists between the three groups of compounds.

In conclusion, after the analysis of several samples of quince jams from several different brands, traditionally and industrially prepared, commercialised in three consecutive years (2000–2002), the phenolics determination is more interesting than that of organic acids and free amino acids, concerning the discrimination of the two types of manufacture. These results indicate that many industrial manufacturers, usually use unpeeled fruits in the preparation of the jams, which is forbidden by Portuguese Legislation.

## Acknowledgements

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# VIII. ACTIVIDADE ANTIOXIDANTE

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Publicação n.º 12 – "Quince (*Cydonia oblonga* Miller) fruit (pulp, peel, and seed) and jam: antioxidant activity"
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J. Agric. Food Chem. 2004, 52, 4705–4712 4705

# Quince (*Cydonia oblonga* Miller) Fruit (Pulp, Peel, and Seed) and Jam: Antioxidant Activity

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To study the antioxidant activity of quince fruit (pulp, peel, and seed) and jam, methanolic extracts were prepared. Each extract was fractionated into a phenolic fraction and an organic acid fraction and was analyzed by high-performance liquid chromatography (HPLC)/diode array detection and HPLC/UV, respectively. Antiradical activities of the extracts and fractions were evaluated by a microassay using 1,1′-diphenyl-2-picrylhydrazyl. The phenolic fraction always exhibited a stronger antioxidant activity than the whole methanolic extract. Organic acid extracts were always the weakest in terms of antiradical activity, which seems to indicate that the phenolic fraction gives a higher contribution for the antioxidant potential of quince fruit and jam. The evaluation of the antioxidant activity of methanolic extracts showed that peel extract was the one presenting the highest antioxidant capacity. The $IC_{50}$ values of quince pulp, peel, and jam extracts were correlated with the caffeoylquinic acids total content. Among the phenolic fractions, the seed extract was the one that exhibited the strongest antioxidant activity. The $IC_{50}$ values of quince pulp, peel, and jam phenolic extracts were strongly correlated with caffeoylquinic acids and phenolics total contents. For organic acid fractions, the peel extract was the one that had the strongest antiradical activity. The $IC_{50}$ values of quince pulp, peel, and jam organic acid fractions were correlated with the ascorbic acid and citric acid contents.

**KEYWORDS:** ***Cydonia oblonga* Miller; phenolic compounds; organic acids; antioxidant activity; DPPH assay**

## INTRODUCTION

In recent years, it has become evident that significant health risks and benefits are associated with dietary food choice (*1*). Fruits and vegetables are rich sources of vitamins, most notably vitamins A and C, and excellent sources of fiber, contain some calories, and are naturally low in fat (*2*). An increased consumption of fruits and vegetables has been associated with protection against various diseases, including cancers and cardio- and cerebrovascular diseases (*3*). This association is often attributed to the antioxidants present in the fruits and vegetables, such as vitamins C and E, carotenoids, phenolic acids, and flavonoids, which prevent free radical damage (*2*).

Quince fruit (*Cydonia oblonga* Miller, Rosaceae family) is a pome with numerous seeds. The fruits are big (10−12 cm in diameter), with variable dimensions and asymmetric shapes, and exhibit a characteristic fragrance. The peel is covered by an abundant hair, which disappears with fruit ripening. The white−yellow pulp, easily oxidized to air exposition, is firm, generally acidic, and astringent; so, it is not suitable for consumption when raw. The most important utilization of this fruit is in the production of jams and jellies, which are very appreciated in Portugal.

Several analytical methods were developed to determine phenolics, organic acids, and free amino acids in quince fruit and jams, and their composition, in terms of these compounds, was established (*4*−*11*). Among these parameters, the phenolic profile determination was revealed to be the most useful in the discrimination of the different parts of quince fruit (pulp, peel, and seed) (*7*, *9*) and in the evaluation of the genuineness of quince puree (*4*), jam (*5*, *6*), and jelly (*12*). Recently, the influence of jam processing upon the contents of phenolics, organic acids, and free amino acids in quince fruit was also evaluated (*13*).

The antioxidant activity of several fruits has been observed in different experimental models (*2*, *14*−*22*). García-Alonso et al. (*22*) analyzed 28 different fruits, including quince pulp, for antioxidant activity determination. Additionally, these authors tried to correlate the antioxidant activity and the flavanol content of these fruits. However, information concerning the antioxidant

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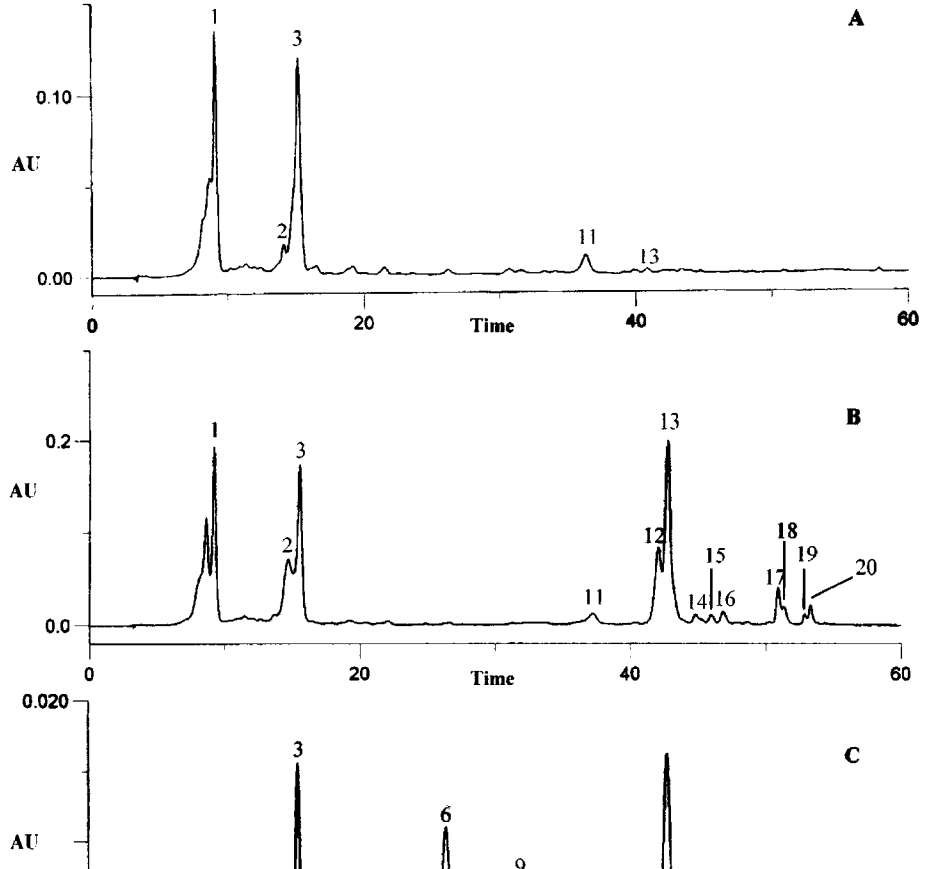



**Figure 1.** HPLC phenolic profile of the quince pulp (**A**), peel (**B**), and seed (**C**). Detection at 350 nm. Peaks: 1, 3-*O*-caffeoylquinic acid; 2, 4-*O*-caffeoylquinic acid; 3, 5-*O*-caffeoylquinic acid; 4, lucenin-2; 5, vicenin-2; 6, stellarin-2; 7, isoschaftoside; 8, schaftoside; 9, 6-C-pentosyl-8-C-glucoside of chrysoeriol; 10, 6-C-glucosyl-8-C-pentoside of chrysoeriol; 11, 3,5-dicaffeoylquinic acids; 12, quercetin 3-galactoside; 13, rutin; 14, kaempferol glycoside; 15, kaempferol 3-glucoside; 16, kaempferol 3-rutinoside; 17 and 18, quercetin glycosides acylated with *p*-coumaric acid; 19 and 20, kaempferol glycosides acylated with *p*-coumaric acid.

potential of quince peels, seeds, and jams is not available. So, in the sequence of previous works and regarding its chemical composition, the purpose of this study was to evaluate the antioxidant potential of quince fruits (pulp, peel, and seed) and jams. To accomplish this aim, the scavenging effect of quince fruit (pulp, peel, and seed) and jam methanolic extracts on 1,1′-diphenyl-2-picrylhydrazyl (DPPH) was studied. The antioxidant activity exhibited by the extracts will be the result of the action of different antioxidant compounds (even from distinct chemical classes) present, with synergies or antagonisms. Considering this, the methanolic extracts were fractionated into the phenolics fractions and the organic acids fractions, which were analyzed by high-performance liquid chromatography (HPLC)/diode array detection (DAD) and HPLC/UV, respectively, and their antioxidant activity was also evaluated. Correlations between the antiradical observed effect and the phenolics and organic acids content were made.

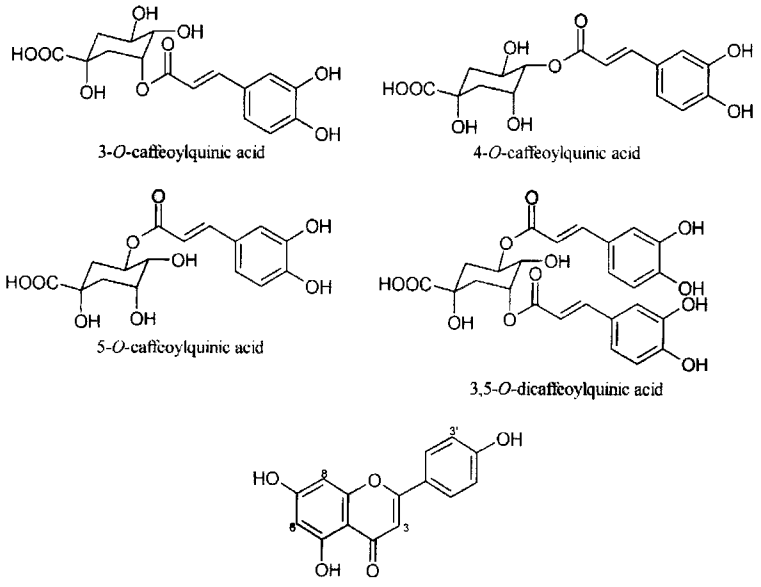
## MATERIALS AND METHODS

**Samples.** Healthy quince fruits were collected in Amarante (Northern Portugal). Some fruits were separated into pulps, peels, and seeds, and each part was freeze-dried. Lyophilization was carried out using a Labconco 4.5 apparatus (Kansas City, MO). Other fruits were used to prepare quince jams.

One quince jam sample (jam A) was prepared in the laboratory by boiling fresh quince pulps with sugar (in the proportion of 50:50), for approximately 90 min. Another quince jam (jam B) was similarly prepared but using unpeeled quinces.

**Standards.** The standards were from Sigma (St. Louis, MO) and from Extrasynthèse (Genay, France). Methanol, formic, and hydrochloric acids were obtained from Merck (Darmstadt, Germany), and sulfuric acid was obtained from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA). DPPH was from Sigma.

**Solid Phase Extraction (SPE) Columns.** The ISOLUTE C18 nonend-capped (NEC) SPE columns (50 $\mu$m particle size, 60 Å porosity;




| Compound | 3 | 6 | 8 | 3' |
|---|---|---|---|---|
| Quercetin 3-galactoside | O-Galactose | H | H | OH |
| Rutin | O-Rutinose | H | H | OH |
| Kaempferol 3-glucoside | O-Glucose | H | H | H |
| Kaempferol 3-rutinoside | O-Rutinose | H | H | H |
| Vicenin-2 | H | Glucose | Glucose | H |
| Isoschaftoside | H | Arabinose | Glucose | H |
| Schaftoside | H | Glucose | Arabinose | H |
| Lucenin-2 | H | Glucose | Glucose | OH |
| Stellarin-2 | H | Glucose | Glucose | $OCH_3$ |
| 6-C-pentosyl-8-C-glucoside of chrysoeriol | H | Pentose | Glucose | $OCH_3$ |
| 6-C-glucosyl-8-C-pentoside of chrysoeriol | H | Glucose | Pentose | $OCH_3$ |

**Figure 2.** Phenolic compounds of quince fruit and jam.

10 g sorbent mass/70 mL reservoir volume) were purchased from International Sorbent Technology Ltd. (Mid Glamorgan, United Kingdom).

**Methanolic Extracts.** Each sample (ca. 1 g for lyophilized pulps, peels, and seeds and 5 g for jams) was thoroughly mixed with methanol (3 × 25 mL) (40 °C). The methanolic extract was filtered, concentrated to dryness under reduced pressure (40 °C) (the extraction efficiency in relation to fresh matter was variable as follows: 17, 14, 10, 89, and 73% for pulp, peel, seed, jam A, and jam B methanolic extracts), and redissolved in methanol (1 mL). These solutions were used for the DPPH assay.

**Organic Acids Fractions.** Each sample (ca. 1 g for lyophilized pulps, peels, and seeds and 5 g for jams) was thoroughly mixed with methanol (3 × 25 mL) (40 °C). The methanolic extract was filtered, concentrated to dryness under reduced pressure (40 °C), and redissolved in acidic water (pH 2.0 with HCl) (ca. 25 mL). The aqueous solutions obtained were passed through an ISOLUTE C18 (NEC) column, previously conditioned with 30 mL of methanol and 70 mL of acidic water (pH 2.0 with HCl). The aqueous extracts were evaporated to dryness under reduced pressure (40 °C) (ca. 30 min) and redissolved in acidic water (1 mL). These extracts were used for the organic acids analysis and DPPH assay.

**Phenolic Compounds Fractions.** After the elution of organic acids and other polar compounds with aqueous solvent, the retained phenolic fraction was eluted with methanol (ca. 50 mL). The extracts were concentrated to dryness under reduced pressure (40 °C) and redissolved in methanol (2 mL). These extracts were used for the phenolic compounds analysis and DPPH assay.

**HPLC Analysis of Organic Acids.** The separation was carried out as previously reported (*8*) with an analytical HPLC unit (Gilson), using an ion exclusion column Nucleogel Ion 300 OA (300 mm × 7.7 mm) column. 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. Malic and quinic acids were quantified together and as malic acid. The other acids were quantified as themselves.

**HPLC Analysis of Phenolics.** The extracts (20 $\mu$L) were analyzed as previously described (*4*−*7*, *12*, *13*), on an analytical HPLC unit (Gilson), using an Spherisorb ODS2 column (25.0 cm × 0.46 cm; 5 $\mu$m, particle size). Detection was achieved with a Gilson DAD.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3-and 4-*O*-Caffeoylquinic and 3,5-dicaffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid. Kaempferol glycoside and kaempferol glycosides acylated with *p*-coumaric acid were quantified as kaempferol 3-glucoside. Quercetin glycosides acylated with *p*-coumaric acid were quantified as quercetin 3-galactoside. The other compounds were quantified as themselves.

**DPPH Method.** 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 of Fukumoto and Mazza (*23*), although some modifications were made to the original DPPH method.

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 $\mu$L of extract and 200 $\mu$L of 150 mM DPPH (dissolved in methanol). The reaction was conducted at room temperature, until

**Table 1.** Phenolic Composition of Quince Pulp, Peel, Seed, and Jams Extracts (mg of Phenolic Compound $kg^{-1}$ of Methanolic Extract Dry Matter)[a]

| | samples | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | pulp | | peel | | seed | | jam A | | jam B | |
| phenolic compounds | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD |
| 3-CQA | 684.6 | 18.86 | 1966.4 | 23.25 | 24.0 | 0.70 | 31.4 | 0.05 | 125.7 | 2.33 |
| 4-CQA | 79.7 | 1.13 | 174.4 | 1.41 | 27.6 | 1.26 | 19.3 | 0.22 | 21.4 | 1.47 |
| 5-CQA | 648.8 | 13.62 | 1829.4 | 31.00 | 54.4 | 1.56 | 72.7 | 0.47 | 128.1 | 0.71 |
| lucenin-2 | ND | | ND | | 10.2 | 1.43 | ND | | ND | |
| vicenin-2 | ND | | ND | | 14.6 | 0.41 | ND | | ND | |
| stellarin-2 | ND | | ND | | 46.6 | 1.01 | ND | | ND | |
| isoschaftoside | ND | | ND | | 17.1 | 0.04 | ND | | ND | |
| schaftoside | ND | | ND | | 11.4 | 0.25 | ND | | ND | |
| 6-C-pentosyl-8-C-glucoside of chrysoeriol | ND | | ND | | 21.8 | 0.12 | ND | | ND | |
| 6-C- glucosyl-8-C-pentoside of chrysoeriol | ND | | ND | | 16.1 | 0.54 | ND | | ND | |
| 3,5-diCQA | 56.3 | 2.60 | 98.7 | 2.41 | 29.9 | 0.17 | 5.4 | 0.23 | 7.5 | 0.17 |
| Q-3-gal | NQ | | 491.2 | 18.86 | ND | | NQ | | 8.7 | 0.43 |
| Q 3-rut | 33.0 | 2.37 | 1777.8 | 27.66 | ND | | 5.3 | 0.33 | 48.8 | 0.32 |
| K-gly | ND | | 112.2 | 0.72 | ND | | ND | | 3.4 | 0.21 |
| K-3-glu | ND | | 88.8 | 2.49 | ND | | ND | | 2.2 | 0.21 |
| K-3-rut | ND | | 152.2 | 1.51 | ND | | ND | | 3.1 | 0.12 |
| Q-gly-*p*-CouA1 | ND | | 166.9 | 1.29 | ND | | ND | | 3.0 | 0.06 |
| Q-gly-*p*-CouA2 | ND | | 65.7 | 0.26 | ND | | ND | | 1.1 | 0.03 |
| K-gly-*p*-CouA1 | ND | | 53.8 | 1.56 | ND | | ND | | 1.3 | 0.01 |
| K-gly-*p*-CouA2 | ND | | 109.5 | 4.77 | ND | | ND | | 2.8 | 0.21 |
| Σ | 1502.4 | | 7087.1 | | 273.6 | | 134.1 | | 357.1 | |
| HMF | ND | | ND | | ND | | 836.8 | 9.16 | 632.0 | 7.25 |

[a] Values are expressed as means of three determinations; SD, standard deviation; Σ, sum of the determined phenolic compound; ND, not detected; NQ, not quantified; jam A, quince jam prepared with peeled fruits; jam B, quince jam prepared with unpeeled fruits; 3-CQA, 3-*O*-caffeoylquinic acid; 4-CQA, 4-*O*-caffeoylquinic acid; 5-CQA, 5-*O*-caffeoylquinic acid; 3,5-diCQA, 3,5-dicaffeoylquinic acid; Q 3 Gal, quercetin 3-galactoside; Q 3 Rut, rutin; K Gly, kaempferol glycoside; K 3-Glu, kaempferol 3-glucoside; K-3-Rut, kaempferol 3-rutinoside; Q-gly-*p*-CouA1 and Q-gly-*p*-CouA2, quercetin glycosides acylated with *p*-coumaric acid; K-gly-*p*-CouA1 and K-gly-*p*-CouA2, kaempferol glycosides acylated with *p*-coumaric acid; HMF, hydroxymethylfurfural.

no variation of the absorbance was observed. Ascorbic acid was used as the reference compound. Four experiments were performed in triplicate.

The antiradical activity was expressed in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% ($IC_{50}$). The $IC_{50}$ value for each extract was determined graphically by plotting the percentage of DPPH scavenging as a function of extract concentration.

## RESULTS AND DISCUSSION

Fruits and vegetables are one of the main sources of antioxidants in our diets (*2*, *14*−*22*). Our previous studies showed that quince fruit is a good source of phenolic acids, flavonoids, and organic acids (*4*−*9*, *12*, *13*), which are considered potent antioxidants (*2*). To test the antioxidant activities of quince fruits and jams, we prepared methanolic extracts of pulps, peels, seeds, and two jams, one of them prepared with peeled quinces (jam A) and another with unpeeled fruits (jam B).

**Identification and Quantification of Phenolic Compounds by HPLC/DAD.** Quince pulp and jam A extracts presented a chemical profile composed of six identified phenolic compounds: 3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic, and 3,5-dicaffeoylquinic acids; quercetin 3-galactoside; and rutin (**Figures 1A** and **2**), which is in accordance with previous studies (*7*, *13*). Quince peel and jam B extracts contained 13 phenolics: the six compounds presented in pulp and jam A extracts plus kaempferol 3-glucoside, kaempferol 3-rutinoside (**Figures 1B** and **2**), and five not totally identified compounds (one kaempferol glycoside, two quercetin glycosides acylated with *p*-coumaric acid, and two kaempferol glycosides acylated with *p*-coumaric acid), as was already observed (*7*, *13*). Like previously described (*9*), the seed extract had a different composition, presenting the referred caffeoylquinic acids plus several flavone *C*-glycosides characteristic of this part of the fruit: lucenin-2, vicenin-2, stellarin-2, isoschaftoside, schaftoside, 6-C-pentosyl-8-C-glucoside of chrysoeriol, and 6-C-glucosyl-8-C-pentoside of chrysoeriol (**Figures 1C** and **2**). In the pulp extract, caffeoylquinic acids represented 98% of the determined phenolics, with 3-*O*-caffeoylquinic acid being the most abundant (46%), while peel extract contained 57% of flavonol derivatives, with rutin being the major one (25%). The peel extract had a higher amount of phenolics than that of the pulp (about five times) (**Table 1**). Caffeoylquinic acids represented 50% of the determined phenolics of seed extract, with 5-*O*-caffeoylquinic acid being the most abundant (20%). This extract contained 50% of flavone C-glycosides, and the major one was stellarin-2 (ca. 17%).

The total flavonoid content of jam A extract was 4%, while that of the jam B was 21% (**Table 1**), a fact that may be explained by the high flavonoidic content of the peel, which was not removed for the preparation of jam B. In quince jam extract chromatograms at 280 nm (data not shown), it was possible to observe a peak corresponding to hydroxymethylfurfural (HMF). The presence of this compound is not strange, once it results from sugar decomposition by heating and cooking duration.

**Identification and Quantification of Organic Acids by HPLC/UV.** As previously reported (*8*), pulp, peel, and jam extracts presented a similar profile composed of seven identified organic acids: oxalic, citric, ascorbic, malic, quinic, shikimic, and fumaric acids (**Figures 3** and **4**). Oxalic acid was the only compound that was not detected in seed extract.

In pulp, peel, and jam extracts, the sum of malic acid plus quinic acid always represented at least 95% of the organic acid content and all other acids were present in very small amounts (**Table 2**). The seed extract was very distinct from the others, in which the sum of malic acid plus quinic acid represented only 33% of the total content (**Table 2**). Citric and ascorbic

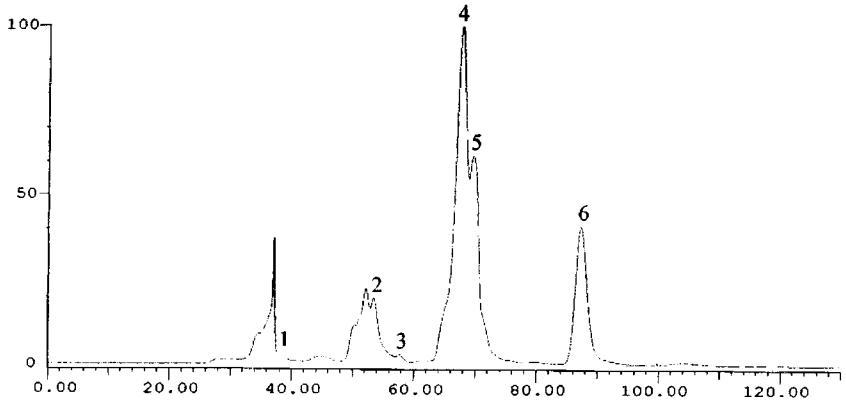



**Figure 3.** HPLC organic acid profile of the quince peel. Detection at 214 nm. Peaks: 1, oxalic acid; 2, citric acid; 3, ascorbic acid; 4, malic acid; 5, quinic acid; 6, shikimic acid.

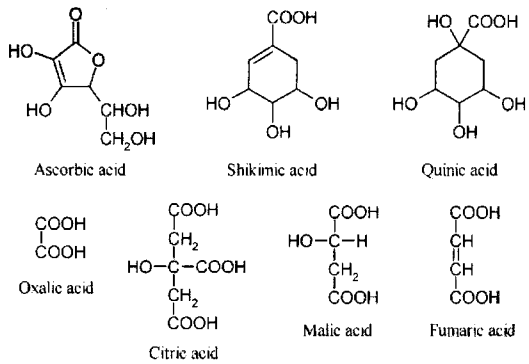



**Figure 4.** Organic acids of quince fruit and jam.

acids were also present in great percentages (36 and 31%, respectively). The organic acid total content of seed extract was the lowest.

**Antioxidant Activity Determination by DPPH Assay.** Once methanolic and acid water extracts (pH 2.0 with HCl) were used, it was necessary to determine the $IC_{50}$ of ascorbic acid solutions (1 mg $mL^{-1}$), dissolved in methanol and acidic water (pH 2.0 with HCl). The ascorbic acid solution $IC_{50}$ value, 5.6 $\mu$g $mL^{-1}$, was not affected by the solvent.

In what concerns the antioxidant activities of methanolic extracts, the peel extract was the one that showed the strongest antioxidant activity ($IC_{50}$ of 0.6 mg $mL^{-1}$), followed by pulp and seed extracts, with very similar activities ($IC_{50}$ of 1.7 and 2.0 mg $mL^{-1}$, respectively) (**Table 3** and **Figure 5A**). Jam A and B extracts also had similar antiradical activities ($IC_{50}$ of 8.9 and 8.4 mg $mL^{-1}$, respectively) (**Table 3** and **Figure 5B**). The results obtained seem to indicate that the $IC_{50}$ of quince pulp, peel, and jam methanolic extracts is correlated with the caffeoylquinic acids total content (exponential decay; $r = 0.99350$; $p < 0.05$). The seed extract exhibited a different behavior, probably because of its different composition, in terms of phenolics (presence of flavone C-glycosides and absence of flavonols) and in terms of organic acids (different individual organic acids percentages and lower organic acid total content).

Among the phenolic extracts, the seed extract was the one that showed the strongest antioxidant activity ($IC_{50}$ of 0.1 mg $mL^{-1}$), followed by the peel extract with an $IC_{50}$ of 0.4 mg $mL^{-1}$ and the pulp extract with an $IC_{50}$ of 1.0 mg $mL^{-1}$ (**Table 3** and **Figure 6A**). Jam A and B extracts had similar antiradical activities ($IC_{50}$ of 7.0 and 6.0 mg $mL^{-1}$, respectively) (**Figure 6B**). The $IC_{50}$ values of quince pulp, peel, and jam phenolic extracts were strongly correlated with the caffeoylquinic acids

**Table 3.** $IC_{50}$ Values (mg $mL^{-1}$), Phenolics, and Organic Acids Total Contents (mg $kg^{-1}$) of Quince Pulp, Peel, Seed, and Jams Extracts[a]

| | methanolic extract | phenolics fraction | | organic acids fraction | |
|---|---|---|---|---|---|
| samples | $IC_{50}$ | total content | $IC_{50}$ | total content | $IC_{50}$ |
| **pulp** | **1.7** | 1502.4 | **1.0** | 16623.7 | 11.6 |
| **peel** | **0.6** | 7087.1 | **0.4** | 14440.5 | 6.9 |
| **seed** | **2.0** | 273.6 | **0.1** | 1858.0 | 12.9 |
| jam A | 8.9 | 134.1 | 7.0 | 4014.0 | 22.6 |
| jam B | 8.4 | 357.1 | 6.0 | 4234.9 | 16.3 |

[a] Jam A, quince jam prepared with peeled fruits; jam B, quince jam prepared with unpeeled fruits.

**Table 2.** Organic Acids Composition of Quince Pulp, Peel, Seed, and Jams Extracts (mg of Organic Acid $kg^{-1}$ of Methanolic Extract Dry Matter)[a]

| | samples | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | pulp | | peel | | seed | | jam A | | jam B | |
| organic acids | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD |
| oxalic acid | NQ | | 5.2 | 0.01 | ND | | 7.2 | 0.01 | NQ | |
| citric acid | **159.0** | **1.11** | 378.2 | 15.17 | 670.2 | 18.76 | 53.8 | 0.24 | 78.3 | 5.97 |
| ascorbic acid | **109.1** | **0.06** | 187.4 | 7.47 | 567.9 | 16.03 | 27.5 | 0.01 | 52.5 | 0.01 |
| malic + quinic acids | 16310.3 | 176.94 | 13818.1 | 66.97 | 611.8 | 7.98 | 3921.3 | 272.76 | 4094.1 | **214.54** |
| shikimic acid | 45.3 | 0.24 | 51.6 | 0.04 | 4.2 | 0.16 | 4.2 | 0.86 | 10.0 | **0.24** |
| fumaric acid | NQ | | NQ | | 3.9 | 0.06 | NQ | | NQ | |
| Σ | 16623.7 | | 14440.5 | | 1858.0 | | 4014.0 | | 4234.9 | |

[a] Values are expressed as means of three determinations. SD, standard deviation; Σ, sum of the determined organic acids; NQ, not quantified; ND, not detected; jam A, quince jam prepared with peeled fruits; jam B, quince jam prepared with unpeeled fruits.

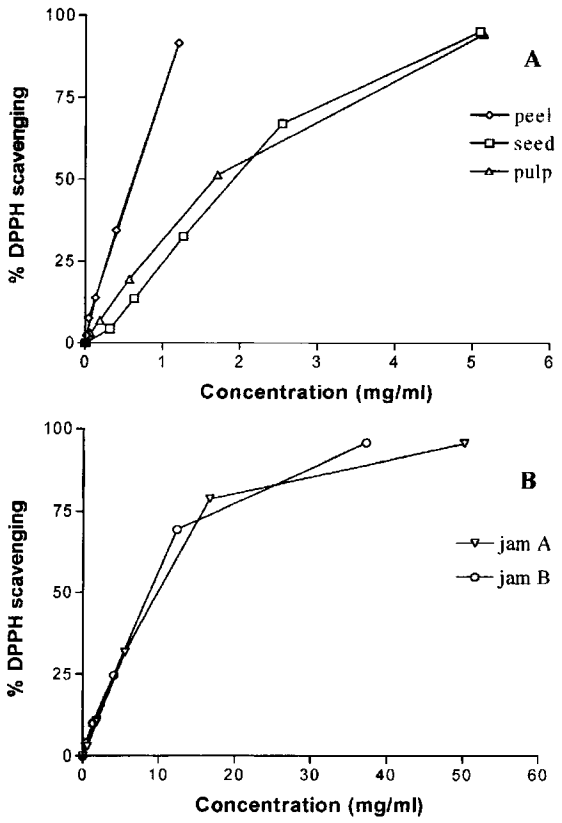



**Figure 5.** Antiradical activity of quince pulp, peel, and seed (**A**) and jams A and B (**B**) methanolic extracts.

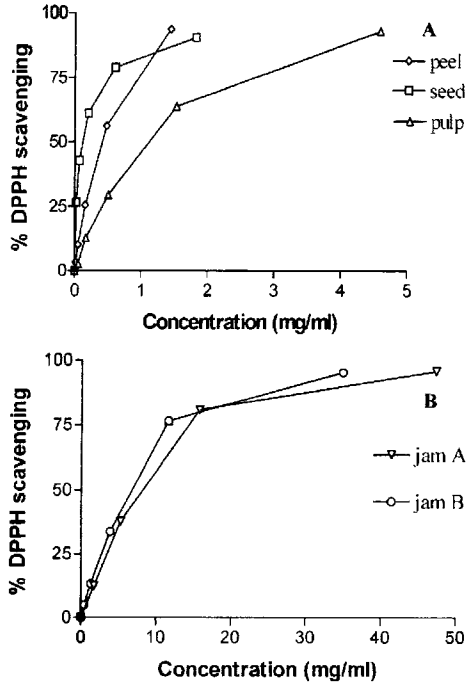



**Figure 6.** Antiradical activity of quince pulp, peel, and seed (**A**) and jams A and B (**B**) phenolic fractions.

total content (exponential decay; $r = 0.99793$; $p < 0.05$) and phenolics total content (exponential decay; $r = 0.99234$; $p < 0.05$). The antioxidant activity of caffeoylquinic acids can be explained by the presence of a catechol group (**Figure 2**), which confers a great stability to phenoxyl radicals by participating in electron delocalization (*24*). Additionally, the conjugated double bond in the side chain of a catechol group is likely to have a great effect in stabilizing the putative phenoxyl radical and, therefore, in enhancing antioxidant activity (*24*). Laranjinha et al. have already reported the antioxidant activity of chlorogenic acid (*24*). Any correlation between $IC_{50}$ and flavonol glycosides total content was not found. These results are in accordance with those of Burda and Oleszek (*25*), who have done a comparison of the antioxidant activity of flavonol aglycons with the activity of its glycosides derivatives and verified that the blockage of the C-3 hydroxyl group resulted in a total loss of antioxidant activity. Glycosylation of other flavonol hydroxyls did not produce such an effect (*25*). The antioxidant activities of quercetin and kaempferol and some of its derivatives have already been reported by some authors (*25*, *26*). Probably, the seed extract had a different behavior because of its different phenolic composition. As previously referred, the seed extract had three C-glycosyl apigenin derivatives (vicenin-2, isoschaftoside, and schaftoside), one C-glycosyl luteolin derivative (lucenin-2), and three C-glycosyl chrysoeriol derivatives (stellarin-2, 6-C-pentosyl-8-C-glucoside of chrysoeriol, and 6-C-glucosyl-8-C-pentoside of chrysoeriol). As can be seen in **Figure 2**, these compounds are characterized by the presence of a hydroxyl group in position 4′ of the B ring, a 2,3-double bond in conjunction with the 4-oxo group in the C ring, and 5,7-dihydroxyl groups in the A ring. This chemical structure determines the radical scavenging effect of flavonoids (*25*, *26*). The presence of the *ortho*-dihydroxy substitution pattern in the B ring, as it happens with luteolin derivatives, is important for the antioxidant activity as well (*26*). Burda and Oleszek (*25*) and Rice-Evans et al. (*26*) have already reported the antioxidant activity of luteolin and apigenin and some of its derivatives. However, the presence of methoxyl substituent in certain positions, as occurs in chrysoeriol, can also increase the antiradical activity of flavonoids (*25*).

Concerning the organic acid extracts, the peel extract was the one that had the strongest antiradical activity ($IC_{50}$ of 6.9 mg $mL^{-1}$), followed by pulp and seed extracts with very similar activities ($IC_{50}$ of 11.6 and 12.9 mg $mL^{-1}$, respectively) (**Table 3** and **Figure 7A**). Jam B exhibited a stronger antioxidant activity than jam A, with $IC_{50}$ values of 16.3 and 22.6 mg $mL^{-1}$, respectively (**Table 3** and **Figure 7B**). The $IC_{50}$ values of quince pulp, peel, and jam organic acid extracts were correlated with the ascorbic acid content (exponential decay; $r = 0.99320$; $p < 0.05$) and citric acid content (exponential decay; $r = 0.98684$; $p < 0.05$). L-Ascorbic acid is a α-keto lactone with an almost planar five-membered ring (**Figure 4**). It has a double bond between the C-2 (or α) and the C-3 (or β) carbons, with the two chiral centers at positions 4 and 5 providing four stereoisomers (*27*). The acidic nature of vitamin C in aqueous solution derives from the ionization of the enolic hydroxyl on C-3, the resulting ascorbate anion being delocalized. The reversible oxidation−reduction with dehydro-L-ascorbic acid is L-ascorbic acid's most important property and the basis for its known physiological activities and stabilities (*27*). Unlike the oxidation−reduction reactions in which ascorbate donates two electrons, the antioxidant reactions use their ability to donate a single electron to free radical species (*27*). Comparing the ascorbic acid content of each organic acid fraction corresponding to $IC_{50}$ (1.3 μg $mL^{-1}$ for pulp and peel extracts, 0.6 and 0.9 μg

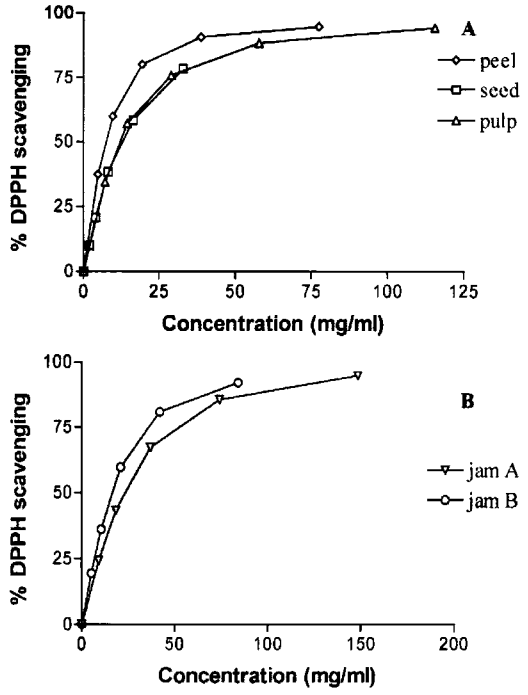



**Figure 7.** Antiradical activity of quince pulp, peel, and seed (**A**) jams A and B (**B**) organic acid fractions.

$mL^{-1}$ for jams A and B extracts) with that of ascorbic acid solutions (5.6 $\mu g$ $mL^{-1}$), it seems that vitamin C was not the only compound that contributed to antiradical activity. In fruits, citric acid protects ascorbic acid from metal-catalyzed oxidation, once it is a chelating agent (*28*). Citric acid functions as a synergist with other antioxidants (*28*). Once the seed extract exhibited great ascorbic and citric acids contents, a lower $IC_{50}$ value was expected, which did not occur probably due to the lower malic and quinic acid contents of this extract, which results in a small organic acid total amount. The ascorbic acid content of the corresponding $IC_{50}$ was 7.3 $\mu g$ $mL^{-1}$, higher than 5.6 $\mu g$ $mL^{-1}$, which may suggest the presence of compounds with prooxidant activity.

Because of the complex compositions of quince fruits and jams, interactions between different antioxidant components are likely important in terms of the overall antioxidant activity of quince fruit and jam. A comparison was made of the antiradical activity of the whole extracts (methanolic extracts) with that of its two fractions. The phenolic fraction always exhibited a stronger antioxidant activity than the whole extracts. Organic acid extracts were always the weakest in terms of antiradical activity, which seems to indicate that the phenolic fraction gives a higher contribution for the antioxidant potential of quince fruits and jams. The antioxidant activities of the analyzed samples cannot only be attributed to their phenolic and/or organic acid contents but to the result of the action of different compounds present in quince fruits and jams and to possible synergic and antagonist effects still unknown. Different amounts and types of minerals can also influence the antioxidant activity of the quince fruits and jams.

In conclusion, this study suggests that phenolic compounds are the main antioxidants in quince. This fruit and its jam can be used as good sources of antioxidants in our diet and may have relevance in the prevention of diseases in which free radicals are implicated. Additionally, quince jam byproducts (peels and seeds) are a good and cheap source of antioxidants, which could be industrially exploited.

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# IX. CONSIDERAÇÕES FINAIS E CONCLUSÕES

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# Considerações Finais e Conclusões

Nesta dissertação de doutoramento realizou-se um estudo aprofundado do marmelo e da marmelada, com vista à sua caracterização em compostos fenólicos, ácidos orgânicos e aminoácidos livres. Para atingir esse objectivo, procedeu-se em primeiro lugar à validação da metodologia de análise dos referidos compostos e em seguida à aplicação dessa mesma metodologia à análise dos compostos em amostras de marmelo de três anos de colheita e de marmeladas comerciais de três anos de comercialização. Após a análise das diversas amostras aplicou-se a PCA aos resultados obtidos. Por fim, avaliou-se o potencial antioxidante de extractos metanólicos de polpa, casca e semente de marmelo e de marmelada, através de um método espectrofotométrico, por monitorização do desaparecimento do radical DPPH.

Os resultados parciais obtidos podem ser vistos nas várias publicações específicas incluídas nesta dissertação. No entanto, parece-nos útil referir alguns dados adicionais e globais para melhor interpretação do trabalho realizado.

## 1. Validação da Metodologia de Análise de Compostos Fenólicos e Respectiva Aplicação às Amostras de Marmelo e de Marmelada

A clássica resina de Amberlite de XAD-2 usada para purificar os extractos fenólicos (eliminar açúcares e outros compostos polares) (Andrade *et al.*, 1998 e 2000; Silva, 1999a, Silva *et al.*, 2000b,c), foi substituída por colunas de extracção em fase sólida Sep-Pack C18 ISOLUTE (NEC), permitindo a obtenção de extractos mais puros, boas recuperações dos analitos, elevada reprodutibilidade, com diminuição considerável do tempo de preparação dos referidos extractos, da quantidade de amostra usada e dos custos (Silva *et al.*, 2001).

A técnica de HPLC/DAD mostrou-se sensível, reprodutível, exacta e adequada para análises de rotina. A preparação melhorada das amostras é simples, envolvendo uma extracção com água ácida (pH 2 com HCl) e purificação através das referidas colunas de extracção em fase sólida. Os limites de detecção foram baixos, entre 0,13 e 1,63 μg/ml, e o método mostrou-se preciso. Geralmente, as recuperações foram elevadas, excepto para a arbutina.

Tornou-se imperioso o recurso a uma técnica de Ion Trap HPLC-DAD-ESI/MS/MS, sem a qual teria sido impossível identificar a maioria dos flavonóides presentes na casca (Silva *et al.*, 2002b) e na semente de marmelo (Ferreres *et al.*, 2003).

O perfil fenólico das três partes do marmelo (polpa, casca e semente) revelou diferenças assinaláveis, quer qualitativamente, quer quantitativamente (Silva *et al.*, 2002b e 2005a). Geralmente, as polpas apresentaram um perfil composto por seis compostos fenólicos: os ácidos 3-*O*-, 4-*O*- e 5-*O*-cafeoilquínicos e 3,5-*O*-dicafeoilquínico, a 3-*O*-galactosilquercetina e a rutina. Em geral, o perfil fenólico das cascas revelou a presença de treze compostos: os seis presentes nas polpas, acrescidos do 3-*O*-glucosilcampferol, do 3-*O*-rutinosilcampferol e de cinco compostos parcialmente identificados (um glicósido do campferol, dois glicósidos da quercetina acilados com ácido *p*-cumárico e dois glicósidos do campferol também acilados com ácido *p*-cumárico).

De uma forma geral, em termos quantitativos verificou-se uma maior abundância do ácido 5-*O*-cafeoilquínico e da rutina nas polpas e nas cascas, respectivamente. A casca apresentou um conteúdo fenólico superior ao da polpa (valores médios de 1176,7 e 224,1 mg/kg, respectivamente), o que não é de estranhar, uma vez que este tipo de compostos, sobretudo os flavonóis, absorve radiação UV sendo, por esse motivo, mais abundantes nas cascas dos frutos (Macheix *et al.*, 1990). Devido às suas propriedades antioxidantes, protegem as células dos danos resultantes da fotoxidação causada pela luz UV (Macheix *et al.*, 1990).

De facto, verificaram-se diferenças muito significativas no teor de todos os compostos fenólicos determinados em polpas e cascas de marmelo ($p < 0,001$). Aliás, a PCA permite uma clara discriminação de polpas e cascas de marmelo. As duas PCs, PC1 e PC2, são responsáveis por 81,29% da variabilidade total. A PC1 (74,14%) revela a diferença entre os conteúdos de ácidos cafeoilquínicos e flavonóides, o que caracteriza a diferença na composição fenólica de polpas e cascas de marmelo, e a PC2 (7,15%) relaciona o conteúdo de ácido 4-*O*-cafeoilquínico com os de ácidos 5-*O*-cafeoilquínico e 3,5-*O*-dicafeoilquínico. A referida discriminação é muito importante do ponto de vista do controlo da qualidade, uma vez que a Legislação Portuguesa não permite a utilização de frutos com casca na produção de marmelada (Decreto-Lei n.º 97/84 de 28 de Março).

O ano de colheita influenciou significativamente o teor em alguns dos compostos fenólicos, o mesmo não acontecendo com a origem geográfica (Silva *et al.*, 2005a).

As sementes apresentaram um perfil fenólico característico, determinado por Ion Trap HPLC-DAD-ESI/MS/MS (Ferreres *et al.*, 2003; Silva *et al.*, 2005b), composto pelos mesmos ácidos das polpas e das cascas e ainda por lucenina-2 (6,8-di-*C*-glucosil luteolina), vicenina-2 (6,8-di-*C*-glucosil apigenina), estelarina-2 (6,8-di-*C*-glucosil crisoeriol), isoschaftósido (6-*C*-arabinosil-8-*C*-glucosil apigenina), schaftósido (6-*C*-glucosil-8-*C*-arabinosil apigenina), 6-*C*-pentosil-8-*C*-glucosil crisoeriol e 6-*C*-glucosil-8-*C*-pentosil crisoeriol.

Enquanto que o perfil fenólico das polpas é caracterizado por grandes percentagens de ácidos cafeoilquínicos e o das cascas por quantidades apreciáveis de ácidos cafeoílquinicos e de diversos glicósidos de flavonóis, o das sementes tem como principal característica a presença de *C*-glicosil flavonas.

Em termos quantitativos, verificou-se uma maior abundância nas sementes de ácido 5-*O*-cafeoilquínico e isoschaftósido (valores médios de 21,25 e 17,67%, respectivamente). O conteúdo fenólico total médio foi de 113,2 mg/kg, sendo inferior aos da polpa e da casca.

Todas as amostras de marmelada apresentaram um perfil abrangendo, pelo menos, os seis compostos fenólicos presentes nas polpas. Contudo, algumas amostras, sobretudo a produzidas industrialmente, também apresentaram os compostos característicos das cascas. A presença destes compostos indica adulteração das marmeladas por utilização de frutos com casca. Geralmente, o composto fenólico mais abundante foi o ácido 5-*O*-cafeoilquínico, com um valor médio de 43,03%.

Verificaram-se diferenças bastante significativas entre os perfis de marmeladas preparadas de forma tradicional e industrial (Silva *et al.*, 2005c). Enquanto que as tradicionais apresentaram um conteúdo superior em ácido 3-*O*-cafeoilquínico ($p < 0,01$), as industriais mostraram um teor mais elevado em flavonóides (3-*O*-galactosilquercetina, $p < 0,001$; rutina, $p < 0,05$; glicósido do campferol, $p < 0,001$; 3-*O*-glucosilcampferol, $p < 0,01$; 3-*O*-rutinosilcampferol, $p < 0,01$; dois glicósidos da quercetina acilados com ácido *p*-cumárico, $p < 0,05$). A PCA permite a distinção clara entre amostras tradicionais e industriais. As duas primeiras PCs caracterizam a composição fenólica (54,38% da variância): PC1 (37,41%) e PC2

(16,97%). A PC1 descreve as diferenças entre os conteúdos em ácidos 3-*O*- e 5-*O*-cafeoilquínicos e em todos os flavonóides; enquanto que a PC2 relaciona os conteúdos de ácidos 4-*O*- e 5-*O*-cafeoilquínicos e os de ácidos 3-*O*-cafeoilquínico e 3,5-*O*-dicafeoilquínico.

A PCA também permite uma discriminação clara entre marmeladas preparadas com frutos com ou sem casca. Os resultados obtidos indicam que a maioria dos produtores industriais usam, frequentemente, marmelos por descascar, o que, de acordo com a Legislação Portuguesa (Decreto-Lei n.º 97/84 de 28 de Março), constitui uma adulteração.

O conteúdo em alguns compostos fenólicos também variou significativamente de acordo com o ano de comercialização e a marca comercial (Silva *et al.*, 2005c).

No decurso do estudo da composição fenólica do marmelo (polpa e casca) e da marmelada (Andrade *et al.*, 1998 e 2000; Silva, 1999a; Silva *et al.*, 2000b, 2001 e 2002b), verificou-se que em todos os cromatogramas registados a 280 nm apareciam alguns picos correspondentes a compostos desconhecidos (compostos **a**, **b** e **c**), com espectro UV idêntico ($\lambda_{máx} \cong 270$ nm), pelo que foi necessário proceder ao seu isolamento e determinação estrutural (Sousa *et al.*, 2005). O isolamento foi efectuado por métodos cromatográficos (CC e HPLC semi-preparativa) e a determinação estrutural realizada recorrendo a métodos espectrofotométricos (UV) e espectrométricos ($^{13}C$ e $^{1}H$ NMR e MS). Foi possível identificar o β-D-glucopiranósido do ácido 9-amino-2,7-dimetil-8-hidroxinona-2,4-dienóico (composto **a**). Este composto e os outros compostos terpénicos aparentados (compostos **b** e **c**) foram descritos pela primeira vez na natureza, podendo vir a revelar-se úteis como marcadores químicos do marmelo e dos seus derivados. Posteriormente, estes três compostos foram quantificados como citral. Geralmente, o teor do composto **a** foi superior ao dos compostos **b** e **c**. Os conteúdos médios dos compostos **a** e **b** de polpa e casca foram idênticos (59,0 e 59,5 mg/kg e 28,9 e 32,3 mg/kg, respectivamente). O composto **c** esteve presente em maior quantidade na casca do que na polpa (24,9 e 14,2 mg/kg, respectivamente). Geralmente, a marmelada apresentou teores inferiores destes três compostos. Contudo, a marmelada U, preparada com polpa obtida a partir de marmelos produzidos em Amarante e colhidos no ano de 2002, apresentou um teor superior no composto **a** do que a respectiva polpa. Este facto reforça a hipótese de que estes compostos possam resultar da degradação dos carotenóides, uma vez que esta pode ser favorecida pelo processamento térmico, a pH ácido natural do marmelo.

Para avaliar os efeitos provocados pelo processamento térmico usado na produção de marmelada, foram preparadas duas amostras de marmelada caseira, uma utilizando polpas de marmelo de Amarante (2002) e outra usando frutos com casca da mesma origem geográfica e do mesmo ano. A proporção de fruto e açúcar utilizada nos dois casos foi de 50:50.

O processamento térmico provocou alguma evaporação, pelo que o conteúdo fenólico total não correspondeu exactamente à percentagem de fruto utilizada, mas sim a uma quantidade superior (57%). Adicionalmente, as altas temperaturas usadas no processamento provocaram alguma isomerização dos ácidos cafeoilquínicos (Silva *et al.*, 2004b).

## 2. Validação da Metodologia de Análise de Ácidos Orgânicos e Respectiva Aplicação às Amostras de Marmelo e de Marmelada

A preparação das amostras é simples, consistindo na extracção com metanol (40ºC) e filtração através de colunas de extracção em fase sólida Sep-Pack C18 ISOLUTE (NEC) (Silva *et al.*, 2002a).

A separação cromatográfica foi efectuada usando uma coluna de exclusão iónica Nucleogel Ion 300 AO (30ºC), recorrendo a eluição isocrática, com uma fase móvel de ácido sulfúrico 0,01 N, com um fluxo de 0,1 ml/min e a detecção foi realizada a 214 nm. Este método mostrou-se preciso, sensível, reprodutível e exacto. Os limites de detecção foram reduzidos, entre 0,01 e 1,67 μg/ml.

As polpas e as cascas de marmelo e as marmeladas apresentaram um diagrama em ácidos orgânicos qualitativo idêntico, abrangendo sete compostos: os ácidos oxálico, cítrico, ascórbico, málico, quínico, shiquímico e fumárico (Silva *et al.*, 2002a e 2005a). O somatório global dos ácidos orgânicos determinados nas polpas e cascas variou entre 2295,8 e 17393,9 mg/kg. O marmelo (polpa e casca) foi caracterizado por quantidades elevadas de ácidos málico e quínico, sendo a sua soma sempre superior a 90% de todos os ácidos orgânicos determinados (valor médio de 96,45%).

Os teores em alguns ácidos orgânicos variaram com o ano de colheita, como aconteceu com os conteúdos em ácido ascórbico ($p < 0,05$), ácido shiquímico ($p < 0,05$) e ácido fumárico

($p < 0,01$) e o teor total ($p < 0,001$). A parte do fruto (polpa ou casca) e a região geográfica de origem não influenciaram significativamente a composição do marmelo em termos de ácidos orgânicos (Silva *et al.*, 2005a).

No que diz respeito ao perfil em ácidos orgânicos das sementes, verificou-se a presença de apenas seis dos sete ácidos presentes nas polpas e nas cascas (Silva *et al.*, 2005b). O ácido orgânico ausente foi o ácido oxálico.

O valor médio do somatório dos ácidos detectados foi de 627,7 mg/kg, sendo bastante inferior ao obtido nas polpas e nas cascas. Para além disso, o valor médio da soma dos ácidos málico e quínico foi de 55,29%, teor bastante inferior ao encontrado para as polpas e cascas, e os ácidos cítrico e ascórbico foram detectados em percentagens apreciáveis, de 24,40 e 19,49%, respectivamente, quando comparadas com as das outras duas parte do fruto (sempre inferiores a 8,42 e 4,76%, respectivamente) (Silva *et al.*, 2005b).

As marmeladas, tal como as polpas e as cascas, foram caracterizadas por elevados teores de ácidos málico e quínico, mas o seu valor médio de 83,17% foi inferior ao encontrado no fruto (Silva *et al.*, 2005c). Além disso, os ácidos cítrico e ascórbico foram detectados em quantidades apreciáveis (valores médios de 14,13 e 2,50%, respectivamente). Muito provavelmente estes resultados devem-se ao facto de estes ácidos serem adicionados nas amostras industriais como regulador de acidez e antioxidante, respectivamente. O conteúdo em ácidos orgânicos variou entre 2514,1 e 12177,5 mg/kg.

Verificaram-se diferenças significativas nos teores de alguns dos ácidos orgânicos presentes em amostras produzidas de forma tradicional e industrial. O ácido cítrico foi mais abundante nas marmeladas industriais ($p < 0,01$), enquanto que a soma dos teores em ácidos málico e quínico foi superior nas tradicionais ($p < 0,01$). O teor em alguns ácidos orgânicos também sofreu influência significativa de acordo com o ano de comercialização e a marca comercial (Silva *et al.*, 2005c).

Todos os ácidos orgânicos presentes no marmelo estão igualmente descritos na maçã e na pêra (Evans e tal., 1983; Coppola & Starr, 1986; Lee & Wrolstad, 1988; van Gorsel *et al.*, 1992; Lal Kaushal & Sharma, 1995; Kadam *et al.*, 1995; Hudina & Stampar, 2000). Por este motivo,

não é possível detectar adulterações por adição destes frutos a marmeladas, através da determinação de ácidos orgânicos, ao contrário da determinação do perfil fenólico.

No estudo da influência do processamento térmico nos ácidos orgânicos verificou-se que, devido a alguma evaporação que ocorre, o conteúdo total dos ácidos orgânicos nem sempre corresponde exactamente à percentagem de fruto utilizada, mas sim a uma quantidade superior. Adicionalmente, as altas temperaturas usadas no processamento provocam alguma degradação dos ácidos cítrico, ascórbico e shiquímico (Silva *et al.*, 2004b).

## 3. Validação da Metodologia de Análise de Aminoácidos Livres e Respectiva Aplicação às Amostras de Marmelo e de Marmelada

A preparação das amostras envolveu uma extracção com água ácida (pH 2,2 com HCl 0,1 M), seguida de um processo de purificação dos extractos com colunas de extracção em fase sólida de troca catiónica forte SCX e uma derivatização rápida dos L-aminoácidos com cloroformato de etilo, uma vez que estes compostos não são suficientemente voláteis para análise directa por GC (Silva *et al.*, 2003).

A separação cromatográfica foi realizada usando uma coluna capilar CP-Sil 19 CB wcot fused-silica, recorrendo a programação da temperatura e da pressão.

Sob as condições escolhidas, foi possível separar os vinte e um aminoácidos livres não só num período de tempo muito curto (6 minutos), mas também com uma boa resolução. A referida metodologia é rápida, sensível, reprodutível e exacta. Os limites de detecção foram muito reduzidos, entre 0,004 e 0,115 μg/ml, e o método mostrou-se preciso, uma vez que os coeficientes de variação dos aminoácidos livres foram baixos.

Devido à sua rapidez e ao seu baixo custo, esta técnica é bastante adequada ao controlo da qualidade de derivados de marmelo.

Apesar das diversas vantagens, este método apresentou a desvantagem de não permitir a determinação da arginina. Durante o processo de derivatização, nem todos os grupos reactivos dos aminoácidos são alterados pela acção do reagente. O grupo imino da arginina permanece inalterado, o que faz com que o derivado deste aminoácido seja absorvido na coluna. Para a

determinação da arginina seria necessária, por exemplo, a sua prévia conversão em ornitina, por acção da arginase.

Todas as amostras (marmelos e marmeladas) apresentaram um diagrama qualitativo de aminoácidos livres idêntico, com vinte e um aminoácidos, mas verificou-se uma grande dispersão em termos quantitativos (Silva *et al.*, 2004a e 2005a,c). Observou-se uma maior abundância neste fruto (polpa e casca) de cinco aminoácidos: a asparagina (20,52%), o ácido aspártico (18,63%), a glicina (12,32%), o ácido glutâmico (11,66% nas polpas e 18,01% nas cascas) e a hidroxiprolina (11,64% nas polpas e 7,82% nas cascas). O teor total em aminoácidos livres variou entre 315,9 e 3113,9 μg/kg nas polpas e entre 454,1 e 2334,6 μg/kg nas cascas.

O perfil em aminoácidos revelou-se similar nas polpas e nas cascas, no entanto, a polpa mostrou-se mais rica em hidroxiprolina e mais pobre em ácido glutâmico ($p < 0,05$) (Silva *et al.*, 2005a).

O teor em alguns aminoácidos livres foi significativamente influenciado pelo ano de colheita e pela origem geográfica (Silva *et al.*, 2005a), o que compromete a utilização deste parâmetro na avaliação da qualidade destes produtos.

No que diz respeito às sementes de marmelo, verificou-se a presença dos mesmos vinte e um aminoácidos, que foram todos quantificados excepto a valina, uma vez que este aminoácido livre co-eluiu com um interferente (Silva *et al.*, 2005b). Esta parte do marmelo revelou um teor total médio em aminoácidos livres de 1550,0 μg/kg, sendo um pouco superior ao das polpas (1274,1 μg/kg) e das cascas (1129,5 μg/kg). Os três aminoácidos livres mais abundantes foram os ácidos glutâmico e aspártico e a asparagina, constituindo entre 60 e 75% do conteúdo total.

O teor total em aminoácidos livres das marmeladas variou entre 309,17 e 2401,31 μg/kg (Silva *et al.*, 2005c). Estes valores podem indicar hidrólise de proteínas, peptídeos e outras moléculas com aminoácidos na sua constituição, hidrólise essa que pode ocorrer durante o processamento térmico (em meio ácido, natural do fruto), tendo em consideração que a percentagem de fruto deste tipo de produtos costuma rondar os 40-50%.

As marmeladas apresentaram dois aminoácidos livres predominantes, o ácido aspártico (32,09%) e a asparagina (30,00%). Verificou-se uma inversão nos conteúdos destes dois

aminoácidos livres em marmelos e marmeladas. Isto pode dever-se ao facto da asparagina ser convertida em ácido aspártico e/ou devido à hidrólise de moléculas com aminoácidos na sua constituição, por acção do calor (em meio ácido).

Verificaram-se diferenças na composição em aminoácidos livres entre marmeladas produzidas do modo tradicional e industrial: teores em ácido glutâmico, glutamina e lisina superiores nas amostras produzidas industrialmente ($p < 0,05$ para os dois primeiros e $p < 0,01$ para a última); teores de ácido aspártico e total mais elevados nas amostras produzidas de forma tradicional ($p < 0,05$ e $p < 0,01$, respectivamente).

O teor em alguns aminoácidos livres também variou significativamente em função do ano de comercialização e da marca comercial (Silva *et al.*, 2005c).

O perfil em aminoácidos livres do marmelo mostrou algumas semelhanças com o da maçã e da pêra (Belitz & Grosch, 1990; Gomis *et al.*, 1990 e 1992; Lea, 1990; van Gorsel *et al.*, 1992), nomeadamente o facto da asparagina e do ácido aspártico serem, quase sempre, os aminoácidos predominantes. Por vezes, tal como na maçã e/ou na pêra, o ácido glutâmico, a cisteína e a histidina estão presentes em maior abundância. Encontraram-se teores de prolina muito baixos (≤ 2,43%), tal como acontece na maçã e ao contrário da pêra (Belitz & Grosch, 1990; Gomis *et al.*, 1990; Lea, 1990).

Apesar das diferenças registadas, a determinação do perfil em aminoácidos livres, ao contrário da dos compostos fenólicos, não permite a detecção de adulterações de marmeladas por adição de pêra ou maçã, uma vez que estes dois frutos não possuem aminoácidos livres cuja presença lhes seja característica.

Ao estudar a influência do processamento térmico no perfil de aminoácidos livres verificou-se que este se alterou de forma considerável, provavelmente devido à hidrólise de proteínas, peptídeos e outras moléculas com aminoácidos na sua constituição, uma vez que as marmeladas apresentaram teores totais em aminoácidos livres superiores aos das polpas e cascas. O teor inferior de ácido glutâmico, histidina e triptofano nas marmeladas, pode dever-se à termolabilidade destes aminoácidos. Outra razão para esta variação pode dever-se à participação destes aminoácidos em reacções de Maillard e/ou de escurecimento após a oxidação enzimática de polifenóis (Silva *et al.*, 2004b).

## 4. Actividade Antioxidante

Uma vez que se verificou que o marmelo é uma boa fonte de ácidos fenólicos, flavonóides e ácidos orgânicos, compostos com reconhecida actividade antioxidante (du Toit *et al.*, 2001), considerou-se do maior interesse avaliar o potencial antioxidante deste fruto (polpa, casca e semente) e da marmelada. Com esse propósito, foram realizados extractos metanólicos de polpa, casca e sementes de marmelos de Amarante (2002) e de duas marmeladas preparadas a partir de frutos da mesma amostra, uma produzida com marmelos sem casca e outra com frutos com casca. O potencial antirradicalar desses extractos foi avaliado recorrendo a um microensaio com o radical DPPH (Silva *et al.*, 2004c). Como a actividade antioxidante dos extractos metanólicos resulta da acção de diversos compostos antioxidantes, de classes químicas distintas, com sinergismos e antagonismos, esses extractos foram divididos em duas fracções: a fracção fenólica e a fracção de ácidos orgânicos.

A escolha deste processo extractivo com metanol deveu-se à necessidade de obter extractos relativamente concentrados, com o menor número de manipulações possível e num período de tempo curto. Esta exigência eliminou a possibilidade de extracção com água, uma vez que a sua evaporação é muito mais morosa do que a do metanol. Assim, a determinação dos aminoácidos livres tornou-se impossível, já que os extractos eram muito pouco puros, o que provocou inúmeras interferências. Por esse motivo, não foi possível correlacionar a composição em aminoácidos livres e o potencial antioxidante dos extractos.

A fracção fenólica apresentou sempre uma actividade antioxidante superior à do extracto metanólico total, enquanto que a fracção dos ácidos orgânicos foi sempre a que revelou uma menor actividade, o que parece indicar que a fracção fenólica é a principal responsável pelo potencial antioxidante do marmelo e da marmelada (Silva *et al.*, 2004c).

De entre os extractos metanólicos totais, o da casca de marmelo foi o que apresentou uma capacidade antioxidante superior. Os valores de $IC_{50}$ dos extractos metanólicos de polpa, casca e marmeladas correlacionaram-se com o conteúdo total em ácidos cafeoilquínicos. O extracto metanólico das sementes apresentou um comportamento diverso, provavelmente devido ao facto

de ter uma composição diferente, tanto em termos de compostos fenólicos (presença de *C*-glicosil flavonas e ausência de heterósidos de flavonóis), como de ácidos orgânicos (conteúdo total inferior e percentagens individuais diversas).

Relativamente às fracções fenólicas, a das sementes foi a que exibiu uma actividade antirradicalar mais forte. Os valores de $IC_{50}$ das fracções fenólicas de polpa, casca e marmeladas estiveram fortemente correlacionados com os conteúdos totais em ácidos cafeoilquínicos e em fenóis.

A actividade antioxidante dos ácidos cafeoilquínicos pode ser explicada pela presença do grupo catecol, o qual confere grande estabilidade aos radicais fenoxilo, participando na deslocalização electrónica (Laranjinha *et al.*, 1994). Adicionalmente, a presença da ligação dupla conjugada na cadeia lateral, tem um efeito estabilizador do radical fenoxilo, o que melhora a actividade antioxidante.

Não se encontrou qualquer correlação entre a actividade antioxidante e o conteúdo em glicosil flavonóis, o que não é de estranhar, tendo em consideração os resultados obtidos por Burda & Oleszek (2001). Estes autores compararam a actividade antioxidante de aglíconas e glicósidos de flavonóis e verificaram que a ligação de um açúcar pelo hidroxilo em C-3 resulta na perda total da referida actividade (Burda & Oleszek, 2001).

Provavelmente, a fracção fenólica das sementes teve um comportamento diferente devido à presença das *C*-glicosil flavonas. Estes compostos são caracterizados pela presença de um grupo hidroxilo na posição 4' do anel B, de uma dupla ligação entre os carbonos C-2 e C-3 em conjugação com o grupo oxo no carbono C-4 do anel C e de dois grupos hidroxilo nos carbonos C-5 e C-7 do anel A. Esta estrutura química determina o efeito sequestrador de radicais livres dos flavonóides (Rice Evans *et al.*, 1995; Burda & Oleszek, 2001). A presença de substituição *o*-dihidroxi no anel B, tal como acontece nos derivados da luteolina, também é importante para a sua actividade antioxidante (Rice Evans *et al.*, 1995). Aliás, Rice Evans *et al.* (1995) e Burda & Oleszek (2001) já descreveram a actividade antioxidante da luteolina e da apigenina e de alguns dos seus derivados. Além disso, a presença de substituições com grupos metoxilo em certas posições, como ocorre no crisoeriol, também pode aumentar a actividade antirradicalar dos flavonóides (Burda & Oleszek, 2001).

No que diz respeito às fracções de ácidos orgânicos, a da casca foi a que apresentou uma actividade antioxidante mais forte. Os valores de $IC_{50}$ destas fracções de polpa, casca e marmeladas estiveram correlacionados com os conteúdos em ácidos ascórbico e cítrico.

O ácido L-ascórbico é uma α-cetolactona com um anel de cinco membros, quase planar, contém uma ligação dupla entre os carbonos C-2 e C-3, com dois centros quirais nas posições 4 e 5, proporcionando a formação de quatro estereoisómeros (Deshpande *et al.*, 1996). A sua natureza ácida em solução aquosa é o resultado da ionização do grupo hidroxilo enólico em C-3, resultando na deslocalização do anião ascorbato. A oxidação-redução reversível com o ácido desidro-L-ascórbico constitui a sua propriedade mais importante, sobretudo no que diz respeito às suas actividades e estabilidades fisiológicas (Deshpande *et al.*, 1996). Ao contrário do que acontece durante as reacções de oxidação-redução, nas quais o ascorbato doa dois electrões, nas reacções antioxidantes o ascorbato oferece apenas um único electrão às espécies radicalares (Deshpande *et al.*, 1996).

Relativamente ao ácido cítrico, este ácido exerce uma acção protectora sobre o ácido ascórbico relativamente aos metais que iniciam os processos de oxidação, devido ao facto de ser um agente quelante (Madhavi *et al.*, 1996). Assim sendo, este ácido vai actuar sinergicamente com outros antioxidantes (Madhavi *et al.*, 1996).

A actividade antioxidante dos extractos analisados não pode ser atribuída unicamente aos seus compostos fenólicos e/ou ácidos orgânicos, mas sim ao resultado da acção global dos compostos presentes nestes extractos de fruto e de marmelada e a possíveis efeitos sinérgicos e antagónicos ainda desconhecidos. Contudo, os resultados sugerem que os compostos fenólicos são os principais antioxidantes do marmelo. Por isso, este fruto e a marmelada podem ser usados como boas fontes alimentares de antioxidantes da nossa dieta, podendo intervir na prevenção de doenças nas quais os radicais livres estão envolvidos. Adicionalmente, os resíduos da indústria da marmelada (cascas e sementes) poderão ser reaproveitados, uma vez que são fontes boas e económicas de antioxidantes (Silva *et al.*, 2004c).

## 5. Nota Final

Ao chegar ao fim deste projecto que me propus desenvolver, para além dos conhecimentos adquiridos e do desenvolvimento intelectual proporcionado, julgo que ele representa um contributo válido na caracterização do fruto de *Cydonia oblonga* e do seu principal derivado, a marmelada.

É também minha convicção que os resultados da aplicação das metodologias aqui apresentadas e uma qualquer casuística em apreço poderão constituir uma mais valia na decisão de confirmar ou de infirmar a respectiva genuinidade de amostras de marmelada, por comparação com os valores médios obtidos neste trabalho. Contudo, são requeridos ensaios adicionais contemplando uma maior amostragem e abrangência para que possam ser definitivos.

A natureza continua a revelar-se complexa e cheia de surpresas que tentamos a todo o custo desvendar. Um pequeno passo foi dado e um sem número de caminhos poderão ser percorridos no sentido de coadjuvar o estudo que aqui se apresenta.

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