12º Encontro de Química dos Alimentos

Composição Química, Estrutura e Funcionalidade: A Ponte Entre Alimentos Novos e Tradicionais

12th Meeting on Food Chemistry

Bridging Traditional and Novel Foods: Composition, Structure and Functionality

Extended Abstracts

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Protective activity of coffee silverskin extracts: evaluation on erythrocyte oxidative-induced hemolysis

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Keywords: Coffee silverskin; Antioxidants; Erythrocytes; Hemolysis; Protective activity.

ABSTRACT

Natural antioxidants from industry by-products have been receiving increasing attention, having in view the sustainability of the processes. Coffee silverskin, a pellicule that covers the raw coffee bean, is a by-product of coffee roasting since it is detached during this procedure. Human erythrocytes are particularly useful in the evaluation of the antioxidant properties of bioactive compounds, since they are susceptible to endogenous oxidative damage due to their specific role as oxygen carriers. The aim of this work was to evaluate the capacity of natural antioxidants of coffee silverskin to protect erythrocytes from oxidative injury induced by the water soluble radical initiator 2,2-azobis (2-amidinopropane)dihydrochloride (AAPH) and by H_2O_2. The results showed that coffee silverskin extracts protected erythrocytes in a significant way from oxidative-AAPH and oxidative-H_2O_2 induced hemolysis. As far as we know, there are no investigations, which evaluate the protective activity of coffee silverskin extracts on human erythrocytes hemolysis.

1.INTRODUCTION

Nowadays, there has been a growing interest in vegetal sources as new therapeutic agents. Food by-products are focus of great interest in scientific community, once they may provide natural antioxidant substances. In addition, valorization and re-use of food by-products minimizes industry wastes, with a higher impact in sustainability and economic concepts.
Antioxidants have natural potential to modulate oxidative stress, which is related to the development of chronic diseases [1,2]. They can also be useful to identify plant species, provide protection to plants, improve the organoleptic properties of vegetable foods, and can be used as natural preservatives against food degradation [3].
Coffee is one of the most traded commodities in the world [4]. Many wastes are associated with coffee roasting and consumption, for instance, coffee silverskin and spent grounds,

respectively [5]. Research on coffee silverskin is limited and mainly directed towards the study of its dietary fiber [6], phenolics content of its extracts, and antioxidant activity (measured by spectrophotometric assays) [2,6,7]. As far as we know, there is no study about the potential of coffee silverskin to protect human cell from oxidative damage in oxidative stress conditions. In the current study, the effect of protective activity of two coffee silverskin extracts on erythrocyte oxidative-induced hemolysis was investigated.

2.MATERIAL AND METHODS

2.1. Samples and extracts preparation

Silverskin samples were supplied by Bicafé - Torrefação e Comércio de Café, Lda., Portugal. The sample chosen for this study represents the major by-product of this industry and resulted from the roast of a commercial coffee blend composed by ~40% of arabica (*Coffea arabica*) and ~60% robusta (*Coffea canephora* var. *robusta*) coffee beans.
After reception, samples were ground (Grindomix GM 200, Retsch, Haan, Germany), homogenized and used for extractions preparation. Briefly, ~1 g of ground sample was extracted with 50 mL of water or ethanol, at room temperature, with constant magnetic stirring (600 rpm) during 60 min. Extractions were performed in triplicate. Extracts were lyophilized and re-suspended in PBS (125 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4) before analysis.

2.2. Preparation of Red Blood Cell (RBC) suspensions

Blood was obtained from four healthy volunteers (two women and two men aged 35-45 years) by venipuncture, and collected into tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Samples were immediately centrifuged at 2000 g for 10 min; plasma and buffy coat were carefully removed and discarded. Erythrocytes were washed three times with PBS at 4ºC and, finally, suspended in PBS to obtain erythrocyte suspensions at 2.6% hematocrit. Erythrocyte suspensions were used in the day they were prepared.

2.3. Hemolysis inhibition evaluation

For the inhibitory evaluation of hemolysis induced by AAPH and H_2O_2, the erythrocyte suspensions were incubated with AAPH or H_2O_2, with different extract concentrations in order to obtain a final concentration of 60 mM of AAPH and 1 mM of H_2O_2, and a hematocrit of 2%. In all sets of experiments (*n*=4), a negative (erythrocyte in PBS) and a positive (erythrocyte in PBS with AAPH or H_2O_2) controls were used. Each control and sample test was analysed in duplicate.
Incubation of RBC suspensions were carried out at 37 ºC for 2 h, under gentle shaking, in the presence of different extract concentrations (40, 20, 10, 2.5, 0.63, 0.16, 0.04, 0.01, 0.0025 and 0.00062 g/L) and initiator radical (AAPH or H_2O_2). In the H_2O_2 assay, sodium azide, in a

final concentration of 1 mM, was added to inhibit catalase activity. Hemolysis was determined spectrophotometrically. After the incubation period (2 h), an aliquot of the erythrocyte suspension was diluted with 20 volumes of saline solution and centrifuged (1200 g for 10 min). The absorption (A) of the supernatant was read at 540 nm. The absorption (B), corresponding to a complete hemolysis, was acquired after centrifugation of erythrocyte suspension that was previously treated with 20 volumes of ice-cold distilled water. The percentage of hemolysis was then calculated (A/B × 100). The percentage of hemolysis inhibition was calculated considering the hemolysis obtained in the positive control tube as 0% of inhibition.

3.RESULTS AND DISCUSSION

Recent studies conducted both in cell cultures and animal models seem to indicate that polyphenols are the main phytochemicals with antioxidant, cardioprotector, anti-inflamatory and antiproliferative properties [8]. Erythrocytes may be considered as major targets to free radical aggression. Thus, oxidative hemolysis of human cells was used as a model to study the antioxidant effect of coffee silverskin extracts on free radicals induced damage of biological membranes, instead of using the *in vitro* spectrophotometric methods for assessing antioxidant activity, such as $DPPH^{\bullet}$ inhibition and FRAP assays. According to our previous report [5], coffee silverskin extracts have high content of total phenolics and flavonoids (8.6 mg GAE/g, 4.3 mg ECE/g, respectively) justifying this study. In what concerns to the inhibition of erythrocyte hemolysis, the results demonstrated that coffee silverskin extracts protected erythrocytes in a significant way from oxidative-AAPH and oxidative-H_2O_2 induced hemolysis, in a dose dependent manner (Fig. 1).

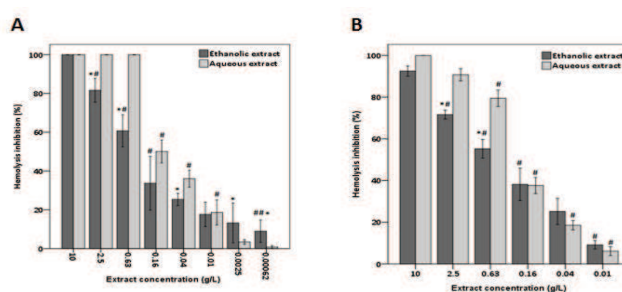

Fig. 1. Inhibition of hemolysis of erythrocytes (mean ± SEM) at 2% hematocrit, by silverskin extracts at different concentrations, incubated with AAPH at 60 mM (**A**) and with 1 mM of H_2O_2 (**B**). # $p<0.05$ *vs* previous concentration; ## $p<0.05$ *vs* 0.04 g/L concentration; * $p<0.05$ *vs* aqueous extract.

As can be observed in Fig. 1, a significant protective effect of silverskin extracts was found at the concentration of 10 g/L, in both methods (AAPH and H_2O_2-induced oxidative stress) and for both type of extracts (ethanolic and aqueous). Moreover, the same results were obtained at concentrations of 2.5 and 0.63 g/L in AAPH-induced oxidative stress biological system using the aqueous extract. At lower concentrations of silverskin extracts, a decrease in the protective activity was observed, being undetectable after 0.00062 g/L and 0.01 g/L in AAPH and H_2O_2-induced oxidative stress biological systems, respectively. When comparing the results

obtained with the two extracts (ethanolic and aqueous), a significant lower protective effect was observed for the ethanolic extract, showing the highest protective activity of the aqueous extracts. The protective effect of more rich silverskin extracts (40 and 20 g/L) were also evaluated. However, given the intensity of dark coloration of silverskin, it was not possible to perform the spectrophotometric readings. Thus, after 2 h of incubation the erythrocyte morphology (Fig. 2) was evaluated, showing a pro-oxidant effect, due to the presence of free hemoglobin erythrocytes (ghost cells).

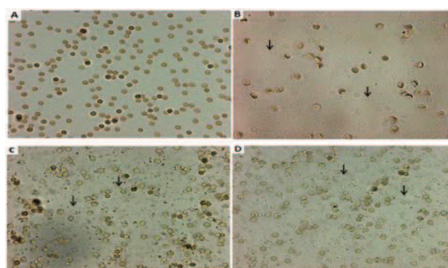

Figure 2. Optical microscopic evaluation of erythrocyte morphology. A) Control just with erythrocytes; B) Positive control with erythrocytes and AAPH, with low cells density and with the presence of free hemoglobin erythrocytes (ghost cells) C) Erythrocytes incubated with 40 g/L of silverskin extract and AAPH, with presence of ghost cells; D) Erythrocytes incubated with 20 g/L of silverskin extract and AAPH, with presence of ghost cells.

4.CONCLUSIONS

The results suggest that coffee silverskin may play an important role in protecting cells against oxidative injuries. Other studies are already being conducted to evaluate the potential of this by-product extract to be integrated in food or cosmetic products.

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Phytochemical analysis of three wild edible Portuguese fruits that may provide potential health benefits

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Keywords: *Arbutus unedo* L.; *Crataegus monogyna* Jacq.; *Rosa canina* L.; phytochemicals; antioxidant activity.

ABSTRACT

Portuguese flora is recognized for its high biodiversity. Many underutilized wild fruits have great nutritional and functional potentials, providing chemical compounds with biological properties. In this work, the phytochemical composition and antioxidant activity of three wild edible fruits (*Arbutus unedo* L., *Crataegus monogyna* Jacq., and *Rosa canina* L.), collected in the North of Portugal, were evaluated. Hydroalcoholic extracts were prepared from fresh fruits, and total phenolics, flavonoids and anthocyanins contents were determined. Moreover, two different assays were used to evaluate antioxidant activity: 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^•$) inhibition assay and ferric reducing antioxidant power (FRAP). As expected, a wide variability was found in the composition of these three wild Portuguese fruits. Phytochemical screening revealed considerable amounts of total phenolics (65-150 mg gallic acid eq./100 g), total flavonoids (126-180 mg epicatechin eq./100 g) and anthocyanins (1.66-29.6 mg cyanidin-3-glycoside eq./100 g). Extracts of *Arbutus unedo* L. fruits presented the highest antioxidant activity, followed by *Crataegus monogyna* Jacq., and *Rosa canina* L., by this order.

1.INTRODUCTION

Wild plants have been receiving increasing attention from the scientific community, due to the diversity of species that can promote a greater variety of edible products (plants, fruits) with recognized medicinal properties. Moreover, it has been suggested that wild food plants can be nutritionally superior to some of the cultivated ones [1].

Arbutus unedo L. (**strawberry tree),** *Crataegus monogyna* Jacq. (hawthorn), and *Rosa canina* L. (**rosa canina fruits)** are some examples of Portuguese natural resources still underutilized [2,3]. Although these fruits have significant importance to local agriculture communities where they are usually used for industrial production of alcoholic beverages,

jam, jellies and marmalades [4], they rarely are eaten as fresh fruits. Nevertheless, their medicinal properties are well described in literature. For instance, the fruits of **strawberry tree** may be employed in folk medicine for antiseptic, diuretic, and laxative effects [5]. Hawthorn fruits are traditionally used to treat high blood pressure, arrhythmia, and digestive disorders [6]. In turn, rosa canina fruits are used in the treatment of cold and other infections, inflammatory diseases, and as diuretic [7].

For all these reasons, the knowledge about the phytochemical composition of these wild edible fruits is of importance to promote their consumption like fresh fruits. The aim of this work was to determine their total phenolics, flavonoids and anthocyanins contents, as well as their antioxidant activity.

2.MATERIAL AND METHODS

2.1. Samples description and extracts preparation

About 3 kg of each type of fruit were collected in the North of Portugal in 2013: hawthorn and rosa canina fruits in August (Porto), and strawberry tree in January (Viana do Castelo). Fruits were homogenized in a blender and used for extracts preparation. Extracts were prepared using ~2.5 g of fruit and 50 ml of a hydroalcoholic solvent (1:1) with magnetic stirring (600 rpm), at 40ºC, during 60 min. After filtration, the final extracts were stored at -25 ºC, till analysis. Extractions were performed in triplicate for each sample.

2.2. Total phenolics

The total content of phenolics in fruit extracts was evaluated according to Costa *et al.* [8]. Briefly, 500 µl of extract were mixed with 2.5 ml of the Folin-Ciocalteu reagent (1:10) and 2 ml of a Na_2CO_3 solution (7.5%, m/v). Subsequently, the solution was incubated during 15 min at 45 ºC, followed by 30 min incubation at room temperature with absence of light. The absorbance was measured in a BioTek Synergy HT microplate reader (GENS5) at 765 nm. A calibration curve was prepared with gallic acid and total phenolics content was expressed as mg of gallic acid equivalents (GAE)/100 g of sample.

2.3. Flavonoids content

Total flavonoids contents were assessed by a colorimetric assay [9]. Briefly, 1 ml of extract was mixed with 4 ml of distilled water and 300 µl of sodium nitrite (25%). After 5 min of incubation at room temperature, 300 µl of 10% $AlCl_3$ were added, followed (1 min after) by 2 ml of sodium hydroxide (1 M) and 2.4 ml of ultrapure water. The absorbance of the mixture was then measured at 510 nm. A calibration curve was prepared with epicatechin and total flavonoids content was expressed as mg of epicatechin equivalents (EE)/100 g of sample.

2.4. Anthocyanins content

Total anthocyanins content was determined according to Burdulis *et al.* [10]. Briefly, an extract aliquot was diluted with 0.1% (v/v) hydrochloric acid and the absorbance of the final solution was measured at 528 nm. Results were expressed as mg cyanidin-3-glycoside equivalents/100 g of sample.

2.5. DPPH$^{\bullet}$ radical scavenging assay

Anti-radical activity of fruit extracts was evaluated by transferring 14 μl of extract (1:10) to 186 μl of a freshly prepared DPPH$^{\bullet}$ solution (9.3 x 10^{-5} mol/L in ethanol). The absorbance decrease was measured at 525 nm to observe the kinetics reaction and a stable plateau was achieved at 40 min. Results were calculated as % of DPPH$^{\bullet}$ inhibition.

2.6. Ferric reducing antioxidant power assay

The analyses were carried out according to the method described by Benzie and Strain [11], with minor modifications. Briefly, 90 μl of diluted extract (1:10) were added to 270 μl of deionized water and 2.7 ml of FRAP solution. After homogenization, the final mixture was held in the dark for 30 min at 37 ºC, and absorbance was subsequently measured at 595 nm. A calibration curve was prepared with ferrous sulfate and reducing antioxidant power was expressed as mg of ferrous sulfate equivalents (FSE)/g of sample.

3.RESULTS AND DISCUSSION

The phytochemical analyses showed the presence of constituents known to exhibit physiological activities that may provide health benefits. Nevertheless, significant differences (p<0.05) were found between the bioactive compounds content of the three wild Portuguese fruits analysed in this study (Table 1).

Table 1. Content of total phenolics (mg gallic acid eq./100 g), flavonoids (mg epicatechin eq./100 g), and anthocyanins (mg cyanidin-3-glycoside eq./100 g) in fruits. Antioxidant activity was measured by DPPH (% of inhibition) and FRAP (mg ferrous sulfate eq./ g) assays.

	Phenolics	**Flavonoids**	**Anthocyanins**	**DPPH**	**FRAP**
Strawberry tree fruits	150 ± 1^{a}	129 ± 2^{b}	1.7 ± 0.2^{c}	83.8 ± 0.3^{a}	115.1 ± 1.6^{a}
Hawthorn fruits	133 ± 3^{b}	180 ± 1^{a}	29.6 ± 1.2^{a}	71.3 ± 0.1^{b}	97.6 ± 3.7^{b}
Rosa canina fruits	65 ± 2^{c}	126 ± 8^{b}	4.2 ± 0.1^{b}	65.2 ± 0.3^{b}	41.7 ± 0.1^{c}

Results are presented as mean ± standard deviation.
Data followed by different letters within each column are significantly different at p<0.05.

Fruits of strawberry tree presented the highest levels of total phenolics, but hawthorn ones showed superiority in flavonoids and anthocyanins contents. **Strawberry tree** fruit

extracts showed also the highest antioxidant activity (in both methods), followed by hawthorn and **rosa canina fruits, by this order.**

Phenolics, due to their hydroxyl groups with scavenging ability, highly contribute to the antioxidant activity of botanical materials. The relation between the total phenolics and total flavonoids contents of the fruits analysed in this study and their antioxidant capacity was determined using linear correlations. There was a good linear correlation (R^2 =0.496 and R^2 =0.941; $p < 0.05$) between the total phenolics content and the antioxidant activity ($DPPH^{\bullet}$ and FRAP, respectively), which was considerably higher than that found for flavonoids and antioxidant activity (R^2 =0.269 for $DPPH^{\bullet}$ and R^2 =0.306 for FRAP assays).

4.CONCLUSIONS

Strong correlations were found between total phenolic compounds and antioxidant activity (especially using the FRAP assay), indicating that total phenolics are the main contributors to the antioxidant activity of these three wild edible fruits.

This study show that these three wild edible fruits present considerable amounts of phytochemicals, with high relevance in their antioxidant activity, and, thus, can provide potential health benefits against free radicals produced in the human body.

Acknowledgments

R. Alves is grateful to FCT for a post-doc grant (SFRH/BPD/68883/2010) financed by POPH-QREN and subsidized by FSE and MCTES. This work received financial support from the European Union (FEDER funds through COMPETE) and National Funds (FCT) through project Pest-C/EQB/LA0006/2013, as well as from FEDER funds under the framework of QREN through Project NORTE-07-0124-FEDER-000069.

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Carotenoid profile of different ready-to-eat baby-leaf vegetables by HPLC-DAD-APCI-MS

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Key-words: baby-leaf vegetables; carotenoid profile; HPLC-DAD-APCI-MS

ABSTRACT

Baby-leaf vegetables are a ready-to-eat products, normally consumed in salads, offering to the consumer a convenient fresh product [1]. Their richness in antioxidant compounds are one of their nutritional advantages, being recognized as a source of bioactive compounds. Carotenoids are plant fat-soluble pigments present in this type of products, which are thought to decrease the risk of degenerative diseases via their antioxidant properties. All carotenoids are capable of inactivating reactive oxygen species (ROS) and may therefore help delay or prevent oxidative damage. However, it is not simple to unequivocally identify the several individual carotenoid compounds present in different plants, due to their diversity and the presence of *cis–trans* isomeric forms.

In this work, the carotenoid profile of several freeze dried samples of baby-leaf vegetables (green and red lettuce, spearmint, watercress and wild rocket) were determined by HPLC-DAD-APCI-MS method. The samples were first extracted with a solution of 50:50 ammonium acetate 10 mM : Methanol (0.1% BHT) and ultrasounds for 15 min to separate the water-soluble compounds. Then, the residue was extracted twice by sonication with ethyl acetate (0.1% BHT) for another 15 min to recover the samples' fat soluble pigments. To identify the carotenoids, each fat-soluble extract was injected in an HPLC-DAD-MS^2 system, being the carotenoids identified based on the comparison with commercial standards retention times and their UV-Vis and MS spectra [1]. As expected, lutein and β-carotene were the major carotenoids found in these samples, followed by the xanthophylls, violaxantin and neoxanthin. The baby-leaf vegetables showed a similar carotenoid profile to the more mature vegetables, being a good choice to the consumer, adding nutritional value and diversity of flavors to their salads.

1.INTRODUCTION

The development of new varieties of new fresh-cut products tries to meet the consumer expectations in terms of convenience, freshness, flavour and quality [1, 2]. The baby-sized

leaves appeared in this market as one of the most promising fresh-cut development, being presented as a natural source of health promoting bioactive phytochemicals [1, 2]. The baby-leaf salads are prepared with young leaves that are washed, mixed, and packaged as whole. Their minimal processing confer the product an attractive presentation, where the leaves keep their 3-D structure and have less signs of oxidation due to a smaller stem diameter [2]. On the other hand, the respiration rate is usually higher in more immature products, which may lead to a faster loss of quality [3]. Carotenoid compounds are one of the major classes of phytonutrients in leafy vegetables, being recognized for their *in vitro* antioxidant capacity [4]. They are terpenoid compounds that contain forty carbon atoms, occurring as pigments usually associated to the photosynthetic apparatus of plants. They are grouped in two main classes: the carotenes (without oxygen atoms, such as lycopene and β-carotene); and the xanthophylls (with oxygen atoms, like lutein, violoxanthin and neoxanthin). In nature, they exist primarily in all-*trans* isomeric form (more stable), being the *cis* isomers less common. The carotenoids function in plants is related to light energy capture, being the most abundant carotenoid compounds in green plants β-carotene, lutein, violaxanthin, and neoxanthin. β-carotene is an important precursor of vitamin A, and all carotenoid compounds are recognized as potent antioxidants. The carotenoid content in plants is dependent of the species and variety, as well as environmental growth factors (such as light, temperature and fertility) and production practices (such as maturity, postharvest handling and storage) [4]. Their chemical structure is also very instable in the presence of light, heat, oxygen and acids, being this a problem to avoid during the extraction process and analysis of carotenoids [4]. The identification of the different carotenoids is normally based on their UV–Vis spectra, being the MS spectra also necessary to differentiate compounds with a very similar UV–Vis spectra [1]. The objective of this work was to characterize and compare the carotenoid composition of five different baby-leaf vegetables stored under refrigerated conditions.

2.MATERIAL AND METHODS

2.1.Samples

Minimally processed green and red lettuce (*Lactuca sativa* var. crispa), spearmint (*Mentha spicata*), watercress (*Nasturtium officinale*) and wild rocket (*Diplotaxis muralis*) were obtained already minimally processed (washed, cut and packed). They were divided in two groups. One was immediately analyzed and the second was stored under refrigerated conditions (3 ± 1 ºC) for 10 days. To preserve the samples until analysis, the baby-leafs were freeze dried, powdered in a knife mill and stored protected from light, oxygen and heat.

2.2. Carotenoids Extraction

About 250 mg of freeze dried sample was extracted with 16 mL of 10 mM ammonium acetate/methanol 1:1 (v/v) in an ultrasound bath for 15 minutes. After centrifugation (14000 g; 15 min) the supernatant was removed. The pellet was then re-extracted twice with ethyl

acetate (0.1% BHT) (6 + 6 mL) in an ultrasound bath (15 min). After centrifuged (14000g, 15 min.) the two supernatants were combined and dried under nitrogen stream. The residue was re-dissolved in 3 mL of ethyl acetate, and filtered through a 0.45 µm nylon filter before the injection in a HPLC-DAD-APCI-MS^2 system.

2.3.Carotenoid Profile Analysis

The equipment used was an Agilent 1200 liquid chromatograph equipped with an autosampler, a DAD, and directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) via an atmospheric pressure chemical ionization (APCI) interface. To separate the compounds an YMC C30 analytical column (5 µm particle size, 250 x 4.6 mm i.d.) was used, together with the following chromatographic conditions: eluents A: MeOH/water (90:10 v/v) and B MTBE/MeOH/water (90:6:4, v/v/v); gradient program, 0 min, 6.5 %B; 8 min, 6.5 %B; 43 min, 100 %B; 46 min, 6.5 %B; 55 min, 6.5 %B; flow rate of 1 mL min^{-1}, injection volume of 10 µL. The DAD recorded the spectra from 220 to 700 nm, and the chromatograms were monitored at 450 nm. MS analysis was conducted in positive ionization mode using the following parameters: capillary voltage, −3.5 kV; drying temperature, 350 °C; vaporizer temperature, 400 °C; drying gas flow rate, 5 L/ min; corona current, 4000 nA; nebulizer gas pressure, 60 psi. A range from m/z 150 to m/z 1300 was acquired and MS/MS automatic mode was used on the more abundant ions. The major carotenoids were identified by combining UV-Vis spectra, chromatographic properties and MS spectra information, with the values obtained from available standards and data reported in the literature. The quantification was made by the construction of two calibration curve, one of lutein to quantify all identified xanthophylls, and one of β-carotene to quantify the carotene isomers. The results were expressed in mg/ 100 g of fresh weight (f.w.), as mean ± standard deviation of two extracts from each sampling day.

3.RESULTS AND DISCUSSION

The used chromatographic conditions allowed the complete separation of the pigments found in the extract obtained from each baby-leaf (Figure 1). The lutein and trans-β-carotene identification was based on the analysis of standards. By its turns, the identification of the other major xanthophylls was based on their characteristic UV–Vis spectra, $[M+H]^+$, and on the different relative intensities of the main fragments obtained by MS^2 experiments (see table 1). The identification of *cis*-β-carotene was confirmed by the presence of a shift of absorption maxima (approximately 4 nm with respect to λ max of trans-β-carotene) and a low *cis* peak at 342 nm.

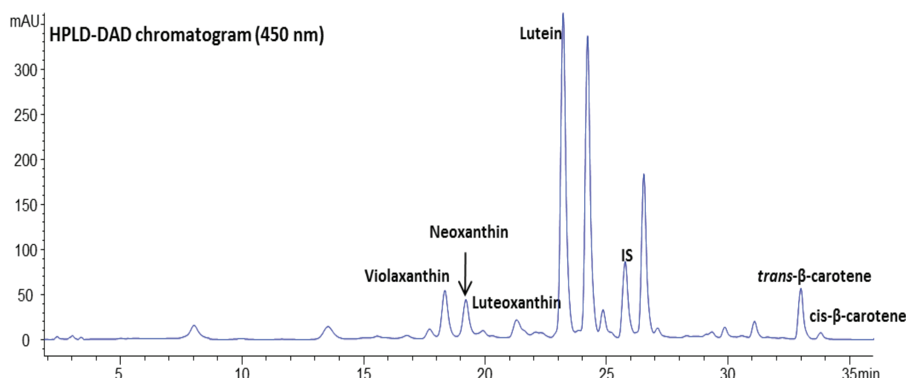

Figure 1. HPLD-DAD chromatogram of the carotenoid profile of spearmint.

Table 1. Retention time (Rt), UV-Vis maxima and mass spectral data of the main xanthophylls found in the baby-leaf vegetables

Carotenoid	Rt(min)	λ max (nn)	$[M+H]^+$ (m/z)	MS^2 main fragment ions (m/z)
Violaxanthin	18.4	416, 440, 469	601.3	583.4, 565.3 509.4, 491.3, 221.0
Neoxanthin	19.3	414, 436, 464	601.5	583.4, 565.4 491.2
Luteoxanthin	20.0	398, 422, 449	601.3	583.4, 491.4, 221.1
Lutein	23.5	423sh, 445, 473	551.4[a]	551.4, 533.4, 495.3,429.3

Regarding the quantification of each carotenoid the results are presented in Table 2. Lutein was the principal compound found in the spearmint, watercress and wild rocket leaves. These leaves also showed a higher mean carotenoid content than lettuce leaves.

Table 2. Individual and total carotenoid content (mg/100g f.w.) of different baby-leaf vegetables (mean value ±standard deviation)

	Lutein	Violaxanthin	Neoxanthin	Luteoxanthin	all–*trans* -β-carotene	*cis* -β-carotene	Total
Green lettuce							
day 1	5.07±0.33	0.76±0.22	1.31±0.12	0.84±0.14	5.21±0.30	1.34±0.23	17.05±3.41
day 10	4.19±0.10	1.39±0.02	1.08±0.02	0.74±0.30	5.30±0.11	1.57±0.04	14.48±4.34
Red lettuce							
day 1	3.43±0.89	0.09±0.00	0.56±0.05	0.14±0.02	3.79±0.97	1.09±0.28	10.41±3.11
day 10	2.14±0.02	0.60±0.15	0.59±0.06	0.19±0.04	3.18±0.02	0.85±0.00	8.72±2.61
Spearmint							
day 1	14.82±1.47	0.34±0.09	1.41±0.25	0.54±0.20	9.89±1.48	3.24±0.18	30.25±3.89
day 10	15.01±0.06	0.54±0.06	1.60±0.01	0.41±0.03	8.79±0.05	3.18±0.02	29.54±0.24
Watercress							
day 1	10.29±0.26	0.88±0.08	1.69±0.06	0.79±0.05	8.48±0.16	2.22±0.04	24.35±0.34
day 10	10.92±0.29	1.37±0.07	1.79±0.01	0.91±0.02	9.14±0.07	2.70±0.08	26.82±0.33
Wild rocket							
day 1	11.53±0.64	1.89±0.46	1.65±0.13	0.90±0.16	8.61±0.36	2.92±0.09	27.50±1.53
day 10	11.64±0.39	1.85±0.05	1.70±0.10	0.98±0.11	9.60±0.89	2.97±0.03	28.75±0.31

4.CONCLUSIONS

The carotenoid profile found in the baby-leaf vegetables was similar to the described for more mature green vegetables. In this sense, these leaves seem to be a good option in terms of the analyzed phytonutrients, being an attractive and convenient way to add nutritional value and diversity of flavors to a balanced meal.

Acknowledgments

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Nutritional quality of commercial bakery products manufactured in Portugal

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Keywords: bakery products; total fat; salt; nutritional quality

ABSTRACT

Bakery products are a wide group of ready-to-eat processed foods, highly consumed, especially by young people. These foods are generally considered as products of low nutritional quality, due to its high content in saturated fat, salt and sugar. The aim of this study was to evaluate the nutritional quality of thirty-one commercial bakery products available in the Portuguese market, considering its total fat and salt content. Moreover, for each product, commercial and supermarket brands were included in the study in order to assess a possible effect of price in the food nutritional quality.

To assure the representativeness of samples, the bakery products were randomly collected, in the major supermarket chains from the Portuguese market. Total fat determination was performed by acid hydrolysis followed by Soxhlet's extraction with petroleum ether. The salt content was determined by Charpentier Volhard's titration. Results of the present study are expressed as g/100 g of edible portion.

Our results showed that bakery products have high total fat contents, varying between 10.2 and 27.7 g/100 g of edible portion. With respect to salt content, the values ranged from 0.0463 to 2.07 g/100 g. Considerable differences in salt contents were found between commercial and supermarket brands.

In summary, this study confirms that bakery products commercialized in Portugal are important sources of fat and salt. Nevertheless, more studies are needed to cover a wider range of bakery products and other processed foods.

1.INTRODUCTION

Bakery products are a wide group of ready-to-eat processed foods, highly consumed, especially by young people. These foods are considered as products of poor nutritional quality, namely due to its high content in saturated fat, salt and sugar. In the past years, food

industry has made efforts to develop food products that at the same time meet the consumers' demand and contribute to a healthier diet. However, the information concerning their nutritional quality is usually scarce and incomplete.
The aim of this study was to evaluate the nutritional quality of thirty-one commercial bakery products available in the Portuguese market, considering its total fat and salt content. Moreover, commercial and supermarket brands for each product were included in the study in order to assess a possible effect of price in the nutritional quality of foods.

2.MATERIALS AND METHODS

2.1.Samples

The samples were selected based on production data and market availability, since up to now, as far as we know, no consumption data is available. Thirty-one samples (filled and unfilled sweet biscuits, sandwich biscuits, brioche with filling, filled croissants, salty snacks, wafers and crackers) of the most common industrial bakery products sold in Portugal were analyzed for their total fat and salt contents. In order to assure the samples representativeness, bakery products were collected randomly, in the major supermarkets chains from the Portuguese market.
At least 6 packages for each sample were randomly collected. Afterwards, three independent composite samples were homogenised in a blender (Grindomix, GM200, RETSCH, Germany) during approximately 1 min at 5000 rpm, in order to provide accurate data that covers possible variations among samples. The results (g/100 g of edible portion) are the average of three individual samples (n=3), each sample analysed in triplicate.

2.2.Salt

The salt content was quantified applying the Charpentier-Volhard's titration method [1]. This method determines the salt content of foods based on the concentration of the chloride ion titrated with silver nitrate solution. Potassium thiocyanate (KSCN, 0.1 N) and silver nitrate ($AgNO_3$, 0.1 N) were prepared in the laboratory. Sodium chloride was estimated from the amount of chloride ion, as determined by the end-point of titration.

2.3.Total fat

Total fat determination was performed according to Albuquerque et al. [2], where an acid hydrolysis followed by extraction using a Soxhlet apparatus (Soxtec™ 2050, Auto Fat Extraction System, FOSS Analytical, Hilleroed, Denmark) with petroleum ether, as the extraction solvent was used. The obtained residue was dried for 1 h 30 min at 101 °C ± 2 °C, until constant weight.

3.RESULTS AND DISCUSSION

3.1.Salt

The total salt content varied between 0.0463 and 2.07 g/100 g of edible portion for brands 12 and 5, respectively (Figure 1). The obtained mean value was 0.705 ± 0.5 g/100 g of edible

portion. WHO recommends a dietary intake lower than 5 g of salt per day for the general population, from all dietary sources, including additives such as monosodium glutamate and preservatives [3]. Most of the analysed bakery products in this study have low levels of salt, but about 19% of the analyzed products contain more than 1 g of salt/100 g.

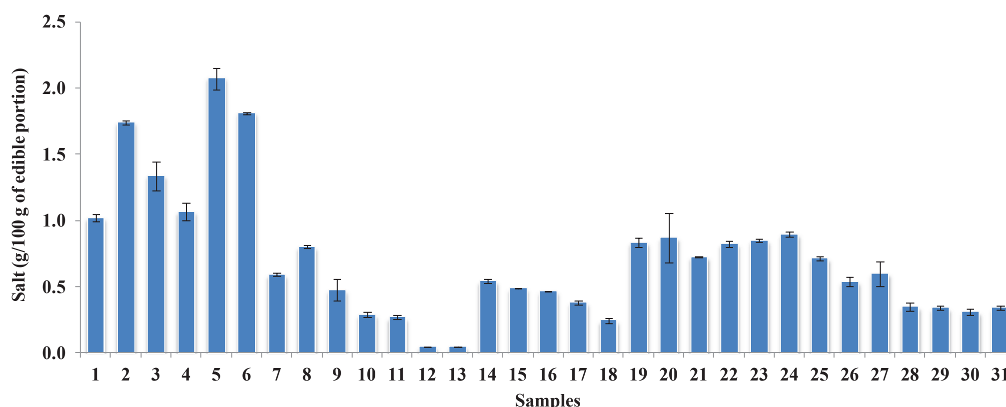

Figure 1. Total salt content of the thirty-one analysed bakery products.

3.2.Total fat

For most European countries, the current recommendation for total fat is a maximum intake value of 30-35% energy (E) per day [4]. Fat is an essential nutrient with several functions, such as, transport of fat-soluble vitamins and for supplying essential fatty acids, which are vital for the formation of signalling substances in the body, known as eicosanoids [5]. As it can be seen from Figure 2, some of the analysed bakery products are fatty dense foods, with a total fat content that varied between 10.2 ± 0.0 and 27.7 ± 1.0 g/100 g of edible portion.

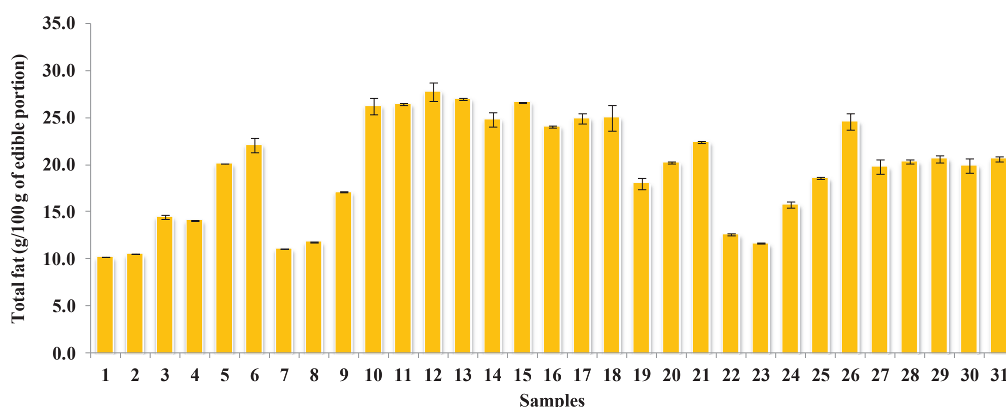

Figure 2. Total fat content of the thirty-one analysed bakery products.

3.3.Commercial vs. supermarket brands

According to the obtained results, the differences with respect to salt content between commercial and supermarket brands were more obvious than for total fat (Figure 3). In this study, the chosen products were separated in eight different food types. However,

supermarket and commercial brands of bakery products were similar for each type. From the thirty-one analysed foods, it was only possible to compare sixteen of those due to the lack of supermarket and/or commercial products. For types 3 and 4 of the analysed bakery products, the supermarket brand has six times more salt content than the commercial brand. It is important to note that these brands had the lowest salt levels. On the other hand, for type 2, commercial brand presented 1.7 times more salt than the supermarket brand.

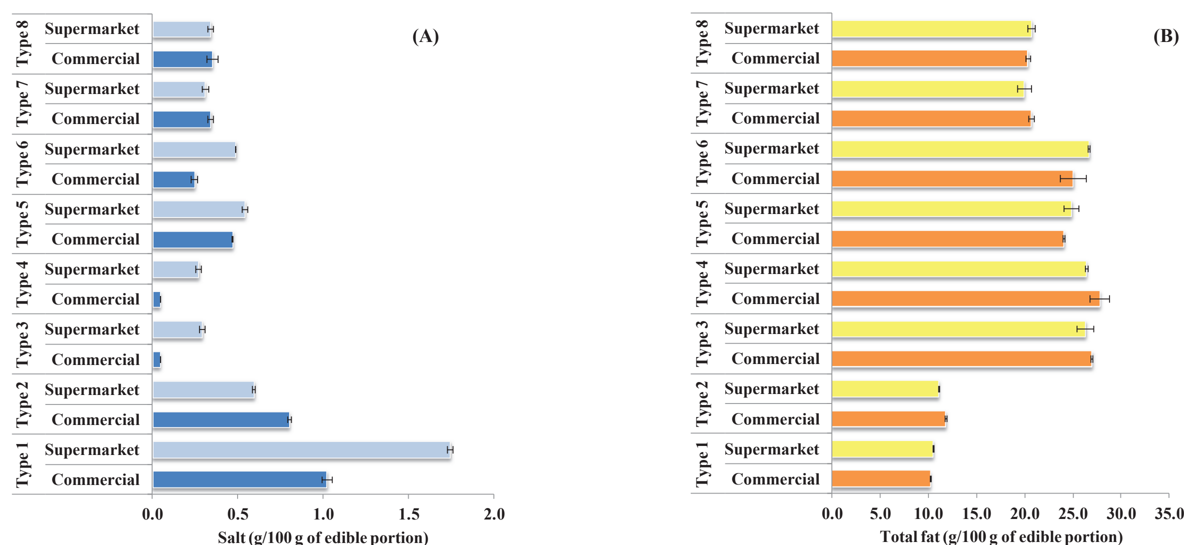

Figure 3. Comparison of commercial and supermarket brands with respect to salt (A) and total fat (B) contents.

4.CONCLUSIONS

In summary, this study demonstrated that bakery products commercialized in Portugal are considerable sources of fat and salt. Since, these foods are highly appreciated, especially by young people, mainly due to the high fat, sugar and salt contents, more studies are needed to cover a wider range of bakery products and other processed foods. Further studies will also evaluate individual fatty acids and cholesterol contents.

Acknowledgements

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Identification of horse meat adulteration by a species-specific mitochondrial DNA marker

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Key words: Species identification; horse meat; qualitative PCR; real-time PCR.

ABSTRACT

Associated with the addition of undeclared horse meat to processed foods, an international scandal has recently emerged, highlighting the need for more strict control of foods. Thus, high sensitive and specific techniques are required to verify labelling compliance, from which DNA-based methods have demonstrated their high potentialities. DNA amplification by polymerase chain reaction (PCR) technique has proved its suitability for unequivocal identification of species in foods.

The aim of this work was to develop a new species-specific PCR assay to detect the addition of horse meat in processed foods. The specificity of the assay was conferred by the design of new primers targeting a mitochondrial region of *cytb* gene to amplify a 141 bp fragment of *Equus caballus*, which allowed the development of two types of assays: species-specific qualitative PCR and real-time PCR with EvaGreen dye. The qualitative PCR results showed a relative limit of detection of 10 mg/kg (0.001%) and 1000 mg/kg (0.1%) in raw and thermally treated binary mixtures, respectively, which decreased to 1 mg/kg by the use of real-time PCR. In terms of absolute detection, sensitivities down to 10 pg and 0.1 pg of horse DNA by qualitative and real-time PCR were achieved for both raw and autoclaved meats. The application of assays to commercial foods revealed the presence of horse DNA in 2 samples acquired before the outbreak of horse meat adulteration becoming known in the EU, emphasising the need to verify labelling compliance of foods.

1. INTRODUCTION

Global incidences of food adulteration are increasing, resulting on disruption of international food trade by frequent disputes over food safety and quality requirements. Concomitantly, to combat fraud and ensure safety and quality, food quality control has been increasing too. Concerning meat products, the focus of adulteration is the partial or total replacement of high valued commercial species by low-valued ones. This results in commercial gain, which may cause serious damage on moral values and on public health. Recently, "the horse meat scandal" arose following the detection of horse meat in beef meat containing foods [1].

However, the presence of horse meat itself does not represent a health risk, unless it has illegally gone into the food chain, containing the veterinary drug phenylbutazone. This drug is not allowed for human consumption because it can cause rare cases of a serious blood disorder like aplastic anemia. Since it is difficult to identify what triggers the anemia, it is not possible to define a safe level of residue in meat. Consequently, horse meat of animals that were medicated with this drug cannot be used as food [2].

Nowadays, the identification of species in food is of major importance to evaluate food authenticity, with special emphasis on meat products. Although the consumption of horse meat is a common practice in many regions of the globe, its undeclared presence might be considered a food fraud against the free consumer's choice about the ingestion of this type of animal. The consumer has the right of an informed choice, which may be a reflection of lifestyles, religious practices or health problems. Therefore, a correct and truthful labelling is crucial to inform consumers about the identity and quality of the food products they are buying. Therefore, analytical techniques with high specificity and sensitivity are needed to verify labelling compliance. The methods based on DNA analysis have proved to be promising tools of high accuracy, emerging as alternatives to protein analysis. The high stability of DNA when compared with proteins, its presence in most of biological tissues and the possibility to specifically amplify short sequences in millions of times by the PCR technique are major advantages [3]. The main objective of this work was to develop a new species-specific PCR assay for the detection of horse DNA in meat products.

2. MATERIALS AND METHODS

2.1 Sampling and DNA extraction

Binary model mixtures were prepared containing horse meat in cow meat in the range of 0.0001% to 25%. In order to assess the effect of heat treatment, a second set of mixtures (with the same concentrations) was autoclaved. To test the specificity of the developed method, 29 samples of plants (wheat, rye, onion, garlic, parsley, white pepper, bay leaf, paprika, chilli, macadamia, walnut, Brazil nut, pistachios, peanuts, fried broad beans with salt, cashews, chestnut, rice, toast pip, sunflower, lupine, almond, oat, barley, rye, soybeans, , hazelnut, tomato cherry and pecan nut) and 15 samples of animal species (boar, duck, partridge, hare, quail, rabbit, pheasant, deer, cow, chicken, turkey, lamb, goat, ostrich and pig), commonly used as foods, were assayed.

DNA was extracted using the Wizard method described by Mafra et al. [4]. Yield and purity of extracts were assessed by UV spectrophotometry using a micro-volume plate accessory.

2.3 Qualitative PCR and real-time PCR

The primers used for this study were designed with software Primer-BLAST designing tool (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/) (Eq-F TCT CAT CCG TCA CTC ACA TCT GC, Eq-R GGA ATG TGT AAG AGC CGT AGT AGA), which was also used to evaluate *in silico* specificity. A mitochondrial region of *cytb* gene of *Equus caballus*

(GenBank accession no DQ297663.1) was targeted with the new primers, enabling to amplify a 141 bp fragment. The specificity of designed *primers* was further confirmed by qualitative PCR.

PCR amplification was carried out in 25 μL total reaction volume containing 2 μL of DNA extract, buffer (67 mM Tris-HCL, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0,01% Tween 20), 2 mM of $MgCl_2$, dNTP (Bioron, Ludwigshafen,Germany), 1 U of SuperHot Taq DNA Polymerase® (Genaxxon Bioscience GmbH, Germany) and 160 nM of each primer (Eq-F/Eq-R). The reactions were performed in a thermal cycler MJ Mini (BioRad, Hercules, CA) with the following temperature programme: initial denaturation at 94°C for 5 min; 35 cycles at 94°C for 30 s, 65°C for 30 s and 72°C for 30 s; with a final extension at 72°C for 5 min.

Real-time PCR assays were performed in 20 μL of total volume, containing 2 μL of DNA, 1x SsoFastTM EvaGreen® Supermix (Bio-Rad Laboratories, EUA) and 160 nM of each primer (Eq-F/Eq-R). The assays were carried out on a thermal cycler CFX96 Real-time PCR Detection System (BioRad, Hercules, CA) using the following conditions: 95°C for 5 min, 44 cycles at 95°C for 15 s and 65°C for 45 s, with collection of fluorescence signal at the end of each cycle. For melting curve data, the temperature was increased by 0.2°C/10 s from 65°C to 95°C. Data were collected and analysed using the software Bio-Rad CFX Manager 3.1 (BioRad, Hercules, CA).

3. RESULTS AND DISCUSSION

The results show that the designed primers allowed the development of a new species-specific PCR assay targeting the *cytb* gene of horse. The assay revealed adequate specificity since no cross-reactivity was observed when testing other plant and animal species commonly used as food. The optimised PCR assay also exhibited high sensitivity, achieving relative limits of detection (LOD) of 10 mg/kg (0.001%) and 1000 mg/kg (0.1%) for raw and thermally treated binary mixtures of horse meat in cow meat, respectively. The absolute LOD was 10 pg of horse DNA, for both raw and autoclaved meats. Therefore, relative sensitivity was altered by thermal treatment, which negatively affects DNA integrity, making it more difficult to detect. The method was successfully applied to 77 commercial food samples, namely 33 acquired before the scandal (2012-2013) and 34 obtained thereafter (2013-2014). Horse meat was detected in two samples prior the scandal, namely a sausage and a hamburger.

To further obtain quantitative results, the same primers were used to develop a real-time PCR assay based on EvaGreen fluorescent dye. The assay enabled decreasing both relative and absolute LOD down to 1 mg/kg (0.0001%) and 0.1 pg (Figure 1), respectively, without being affected by thermal processing. To develop a robust quantitative methodology, both species-specific and endogenous control primers should be used in combination. The use of an endogenous control is of utmost importance for quantitative purposes, especially when processed products with complex composition are considered. Therefore, the parallel amplification of both species-specific and endogenous control genes enabled the

development of a quantitative calibration model based on ΔCt method in the range of 25-0.0001%. The real-time PCR performance parameters were according to the definition of minimum performance requirements [5] since the correlation coefficient (R^2) of standard curves was above 0.98 and PCR efficiency between 90% and 110%, which implicates slopes ranging from -3.6 and -3.1, respectively. The method was effectively validated with blind meat mixtures from 15% to 0.25% with relative errors <20%. The application of the quantitative model to the 2 positive samples showed that both of them contained trace amounts of horse DNA (0.19% – sausage, <LOD – hamburger). These findings suggest that the presence of horse might be due to cross-contamination in the production line and not as the result of a fraudulent addition.

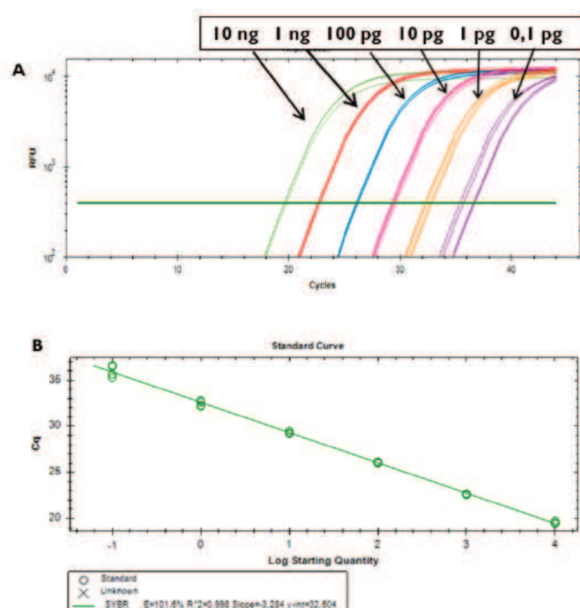

Figure 1. Amplification curves (A) and respective calibration curve (B) obtained by real-time PCR of a horse DNA extract serially diluted for the determination of absolute LOD.

4. CONCLUSION

In the present work, two methods were successfully developed for the detection and quantification of horse species (*Eqqus caballus*) that showed high sensitivity and specificity. It is possible to conclude that, through the development of this work, new useful and effective tools were proposed for the analysis of adulteration of horse meat in meat products.

Acknowledgments
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Development and validation of a voltammetric biosensor for the detection of a major peanut allergen in foodstuffs

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Keywords: Ara h 1; immunosensor; voltammetry; peanut; allergen

ABSTRACT

A gold-nanostructured voltammetric immunosensor for Ara h 1 (a major peanut allergen) analysis was developed, validated, and used to detect its presence in food samples.
Gold nanoparticles were electrochemically deposited on the surface of screen-printed carbon electrodes. A sandwich immunoassay, using two monoclonal antibodies, in which the antibody-antigen interaction was detected through voltammetric stripping of enzymatically deposited silver (using alkaline phosphatase and silver ions) was developed.
The proposed immunosensor provided precise and accurate results and presented very low limits of detection and quantification (3.8 and 12.6 ng/ml, respectively). Moreover, it was able to detect very small amounts of Ara h 1 (0.1%) in a complex food matrix (cookies).

1.INTRODUCTION

Food allergies, which affect up to 10% of the general population, have been increasing in industrialized countries. These allergies arise mainly from an immunological hypersensitivity, usually against certain food proteins or glycoproteins (antigens), mediated by allergen-specific immunoglobulin E (IgE). Clinical manifestations include digestive disorders, respiratory symptoms, circulatory problems, skin injuries, and, in some cases, life-threatening reactions (anaphylactic shock). Food allergies are usually controlled by allergen avoidance (very small amounts of allergen, from less than 1 mg to some grams, can cause an allergic reaction in a sensitized person) or treatment of the symptoms [1,2].
In the European Union, Directive 2000/13/EC, as amended by Directives 2003/89/EC and 2007/68/EC, establishes a list of several food ingredients that must be indicated on the label of foodstuffs because they are likely to cause adverse reactions in susceptible individuals. For these individuals a correct and assertive labeling concerning the presence of potential allergens in foodstuffs is extremely important. However, cross-contamination during food processing or ingredients with "hidden" allergens can turn this into a difficult task. Therefore, food manufacturers usually decide to present a preventive label ("may contain traces of...") not only to protect sensitized individuals but also themselves. This reduces the available

options of food for allergic consumers. Moreover, in a perspective of quality control and improvement of existing food safety systems (e.g. HACCP, ISO 22000:2005), the development of new methodologies (fast, reliable, accurate, and highly sensitive and selective) which are able to detect vestigial amounts of allergens in foods (complex matrices) is crucial [2].

Peanut (*Arachis hypogaea*) is one of the most allergenic foods. Ara h 1 (a major peanut allergen) is a seed storage protein, and is thermostable and resistant to digestion in the human gastrointestinal tract, being recognized by serum IgE from more than 90% of peanut-allergic patients. For these reasons, it is considered a suitable marker for the identification of peanut presence in food products and production lines [3].

In this work, a gold-nanostructured voltammetric immunosensor for Ara h detection and quantification was developed, validated, and used to analyze different food samples.

2.MATERIALS AND METHODS

2.1.Instrumentation and reagents

The electrochemical procedures were performed using an Autolab PGSTAT12 potentiostat-galvanostat from Metrohm Autolab controlled by GPES4.9 software (Metrohm Autolab). Screen-printed carbon electrodes (SPCEs) were purchased from DropSens. A Heraeus Fresco 17 Centrifuge (Thermo Fisher Scientific) was used for samples preparation.

Tris(hydroxymethyl)aminomethane (Tris), magnesium nitrate hexahydrate, hydrochloric acid, nitric acid, β-casein, 3-indoxyl phosphate disodium salt (3-IP), streptavidin-alkaline phosphatase (S-AP), and sodium chloride were all obtained from Sigma-Aldrich. Silver nitrate was purchased from Alfa Aesar and tetrachloroauric(III) acid was supplied by Merck. Mouse monoclonal anti-Ara h 1 IgG, biotinylated mouse monoclonal anti-Ara h 1 IgG and Ara h 1 were obtained from Indoor Biotechnologies. Skimmed milk (Nestlé) was bought in a local supermarket.

2.2.Samples and sample preparation

Cookies, chocolates and peanuts were bought in local supermarkets. Cookies containing increasing amounts of peanut (0.1, 0.3, and 1.5%) were prepared using the cookie matrix as base.

After homogenization, 1g of sample was extracted with 10 ml of Tris–HNO_3 buffer (pH 8.2), at 60ºC, during 30 min. For chocolate samples, 1 g of skimmed milk was added before extraction. Samples were subjected to subsequent centrifugation steps. An aliquot (40 μl) of the final supernatant was used for the immunosensing assay. Dilutions were performed when necessary.

2.3. Sensor fabrication and immunoassay

In situ electrochemical deposition of gold nanoparticles (NPAus) on the SPCEs was performed by applying a constant current intensity of -100 µA for 240 s, followed by the application of a potential of 0.1 V during 120 s [4]. The SPCE–NPAus working electrodes were coated with 10 µl of an anti-Ara h 1 antibody solution and left to incubate overnight at 4ºC. After incubation, the electrode was washed with 0.1 M Tris–HNO_3 pH 7.2 buffer and free surface sites were blocked with β-casein during 30 min. The analysis of Ara h 1 was achieved by incubating the immunosensors, after washing with 0.1 M Tris–HNO_3 pH 7.2 buffer, with an Ara h 1 solution or a food sample extract for 60 min. Then, the biosensor was washed again with the 0.1 M Tris–HNO_3 pH 7.2 buffer and incubated with a previously prepared mixture of biotinylated monoclonal anti-Ara h 1 antibody and S-AP for 120 min. After rinsing, the enzymatic reaction was carried out by placing 40 µl of a solution containing 3-IP (1.0×10^{-3} M) and silver nitrate (4.0×10^{-4} M) on the immunosensor's surface. In this step, AP hydrolyzes 3-IP resulting in a indoxyl intermediate that reduces the silver ions in solution resulting in metallic silver and "indigo blue". After a 20-min reaction, a linear sweep voltammogram was recorded from −0.02 V to +0.40 V, at a scan rate of 50 mV/s, to obtain the electrochemical oxidation current of the enzymatically deposited silver [5].

3.RESULTS AND DISCUSSION

In order to select the more effective concentrations of both capture and detection antibodies, different conditions were tested. First, for a fixed concentration of capture antibody, different dilutions of detection antibody were tested (1:500, 1:1000, 1:5000, 1:10000). The three first dilutions (1:500, 1:1000, 1:5000) resulted in higher peak current intensities, however, the respective blank signals were significantly higher ($p<0.05$) than those obtained with a 1:10000 dilution. Because low blank signals are analytically preferable, the 1:10000 solution was selected to proceed the studies. Subsequently, for this detection antibody dilution, different concentrations of capture antibody were tested (5, 10, 25, 50 and 100 µg/ml). The best results were obtained using 25 µg/ml: a significantly higher ($p<0.05$) peak current intensity in the presence of the allergen and, simultaneously, low blank signals.

Then, different formats were studied in order to reduce the steps and/or the total time of the immunoassay. For that, different combinations of immunoreagents as well as distinct incubation times were tested. The best results were achieved by combining detection antibody and S-AP 60 min before a 120-min incubation on the sensor surface.

The precision (intra- and inter-day) of the results obtained with the developed immunosensor were 2.9% and 8.7%, respectively. Linearity between peak current intensity and Ara h 1 concentration was established between 25 and 2000 ng/ml ($y=(17.89\pm1.66)\times[\log C]-(24.34\pm1.81)$, $r=0.997$, $n=4$, where "y" is the peak current intensity in µA and "C" is the Ara h 1 concentration in ng/ml). The limits of detection and quantification were 3.8 ng/ml and 12.6 ng/ml, respectively.

For recovery experiments, a cookie sample extract was spiked with four levels of known concentrations of Ara h 1 (50, 250, 500 and 1000 ng/ml). The obtained recoveries were 98.9, 98.7, 97.7, and 96.6%, respectively. In the absence of a reference material, pure peanut beans were extracted, in triplicate, and Ara h 1 protein was quantified. The found value of 12.76±1.65 mg/g (n=3) is in good agreement with the concentration of Ara h 1 in peanut described in the literature [6].

Spiked samples containing increasing amounts of peanut (0.1, 0.3, and 1.5%) were prepared using the cookie matrix as base. Results showed that even the lowest amount (0.1%) of peanut could easily be detected with this immunosensor. Ara h 1 presence was also evaluated in commercial chocolate samples with and without peanut (selected based on their label information). The chocolate sample without peanut gave a negative result (no significant differences (p>0.05) when compared to the blank assays). In the chocolate sample containing peanut as ingredient, 8.7 µg of Ara h 1/g of chocolate were detected.

4.CONCLUSION

A gold-nanostructured screen-printed carbon electrode was used to develop a two-monoclonal antibody sandwich-type immunosensor for Ara h 1 detection. Electrochemical detection was based on AP-catalyzed metal precipitation. Optimization of variables involved in the immunosensing strategy was performed, namely regarding the optimum concentrations of each antibody, the number of the steps to perform analysis, and the time of the assay. Validation parameters indicate that the proposed methodology provides accurate and precise results with a very low limit of detection (3.8 ng/ml). The immunosensor was successfully applied to Ara h 1 detection in complex food matrices, such as cookies and chocolate.

Acknowledgments

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Nutritional profile of edible marine macroalgae: macronutrients

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Keywords: Macroalgae; sustainability; nutritional profile.

ABSTRACT

In this study, the nutritional profile of widely consumed species of seaweed (commercially available in the Portuguese market) was evaluated. Three brown macroalgae, *Undaria pinnatifida* (Wakame), *Laminaria japonica* (Kombu) and *Hizikia fusiforme* (Hijiki), and three red ones, *Palmaria palmata* (Dulse), *Porphyra tenera* (Nori) and *Eisenia bicyclis* (Arame), from different geographical origins (Japan and Galiza, Spain) were analysed. The chemical analyses were performed to determine moisture, ash, protein, fat and carbohydrate contents.
The algae samples are commercialized dry, therefore, the moisture content was very low (~10%). The ash content was around 17-38% in brown algae and 10-17% in red algae. Protein levels ranged from 6 to 33%. The highest levels were found in red species, especially in *Porphyra tenera*. The fat content was less than 1%. The carbohydrate content, which includes the dietary fibre, ranged from 38 to 69%. The results of this work show that the analysed macroalgae can be interesting alternative sources of protein, minerals, and, eventually, fibre.
Further studies are being conducted in order to better characterize these species in what concerns to their chemical composition and potential health effects.

1. INTRODUCTION

Water covers about 70% of Earth's surface. The oceans are a source of biological diversity, being the habitat of thousands of plants, animals and different types of microorganisms that contribute for about 50% of the global biodiversity [1]. Marine biodiversity represents, indeed, an exceptional source of natural resources. Their use in a sustainable way may ensure alternative food sources for human consumption, which scarcity is being anticipated.
Macroalgae, also known as seaweed, are an outstanding example of this biodiversity and an excellent source of a wide number of chemical compounds with beneficial health effects [2]. According to their pigmentation, they can be distinguished in green (*Chlorophytaea*), brown (*Phaeophytaea*) and red (*Rhodophytaea*), showing significant differences in nutritional and chemical compositions [3]. Some macroalgae are widely used as food ingredients in oriental

countries, as good sources of fibre and protein [4]. They are also considered nutraceuticals, with several health benefits described in literature, such as anti-inflammatory, anti-allergic, antimutagenic, antitumor, antidiabetic, antihypertensive, and neuroprotective [2,5]. For these reasons, macroalgae are a very attractive material for food, pharmaceutical and cosmetic industries.
The aim of this study was to analyse the nutritional profile of 6 widely consumed species of macroalgae (3 brown and 3 red), from different geographical origins (Japan and Galiza, Spain), commercially available in the Portuguese market.

2. MATERIALS AND METHODS

2.2.. Chemicals

Sulphuric acid, sodium hydroxide, boric acid, Kjeldahl tablets and anhydrous sodium sulphate were of analytical grade from Merck (Darmstadt, Germany). Petroleum ether was of analytical grade from Sigma (ST Louis, MO, USA).

2.3. Samples and sample preparation

Dried seaweeds were purchased in specialty shops in Porto. For analysis, all samples were ground (Grindomix GM 200, Retsch, Haan, Germany) and homogenized. Samples were properly placed in sample containers until analyses.

2.4. Moisture

Moisture determination was performed using a moisture analyser (Scaltec SMO 01, Scaltec Instruments, Germany), at 105 ºC.

2.5. Ash contents

The ash content was determined according to AOAC 950.153 [6], by placing ground samples in a muffle furnace (Thermolyne 48000, Electrothermal Engineering Ltd, Essex, United Kingdom) at 500 ºC for 24 h.

2.6.. Protein contents

Total protein content was determined by the Kjeldahl method (AOAC 928.08) [6]. Approximately 0.5 g of seaweed were digested in an Automatic Digestor K-438 (Büchi®, Büchi Labortechnik AG, Switzerland) for 2 hours with concentrated sulphuric acid and two Kjeldahl catalyst tablets. After digestion, the distillation was performed using a Kjeldahl Distilling Unit K-360 (Büchi®, Büchi Labortechnik AG, Switzerland). The boric acid solution receiving the distilled ammonia changed colour from red to green. After that, the solution was titrated against standard sulphuric acid until yellowish end point. The conversion factor used was 6.25.

2.7. Total lipid contents

The lipid content was determined using the Soxhlet method (AOAC 991.36) [6]. Approximately 5 g of sample was homogenized with anhydrous sodium sulphate. Lipids were extracted with petroleum ether 40 - 60 ºC for 8 hours.

2.8. Total carbohydrate contents

The carbohydrates were calculated indirectly according to the following equation:

% total carbohydrates = 100% - (% moisture + % ash + % protein + % lipids)

3. RESULTS AND DISCUSSION

The algae samples are commercialized dry so the moisture content was very low, around 10% (Table 1). The ash content, which includes the mineral fraction, varied from 17 to 38% in brown algae and 10 to 17% in red ones.

Table 1. Moisture, ash, protein, lipids and total carbohydrates contents of some edible seaweeds (contents are expressed in %).

Samples	Moisture	Ash	Protein	Lipids	Carbohydrate
L. japonica [g]	10.57 ± 0.40	31.00 ± 0.26	5.82 ± 0.04	0.47 ± 0.00	52.14 ± 0.62
L. japonica [j]	14.00 ± 0.15	23.98 ± 0.05	9.32 ± 0.04	0.80 ± 0.02	51.91 ± 0.23
U. pinnatifida [g]	9.89 ± 0.83	37.92 ± 0.55	13.56 ± 0.04	0.85 ± 0.06	37.78 ± 0.27
U. pinnatifida [j]	12.12 ± 0.26	30.43 ± 0.25	12.74 ± 0.05	0.49 ± 0.04	44.22 ± 0.09
H. fusiforme [j1]	12.96 ± 0.30	17.08 ± 0.13	7.95 ± 0.01	0.60 ± 0.06	61.42 ± 0.12
H. fusiforme [j2]	9.82 ± 0.29	17.97 ± 0.13	8.69 ± 0.06	0.31 ± 0.03	63.21 ± 0.13
P. tenera [g]	12.78 ± 0.68	9.51 ± 0.02	29.90 ± 0.01	0.22 ± 0.01	47.60 ± 0.66
P. tenera [j]	6.80 ± 0.67	10.33 ± 0.21	33.33 ± 0.07	0.47 ± 0.00	49.07 ± 0.52
E. arborea [j]	9.18 ± 0.64	11.80 ± 0.08	9.53 ± 0.00	0.39 ± 0.00	69.10 ± 0.57
E. bicyclis [j]	7.98 ± 0.05	12.18 ± 0.02	12.12 ± 0.09	0.56 ± 0.03	67.16 ± 0.00
P. palmata [g]	10.78 ± 0.38	17.06 ± 0.02	17.70 ± 0.03	0.11 ± 0.00	54.35 ± 0.39

Mean values of triplicate determinations ± standard deviations.
g, algae from Galiza; j, algae from Japan.

Protein levels ranged from 6 to 33% and the highest content was found in red species, especially in *Porphyra tenera*. The fat content was less than 1% in all species (0.11-0.85). The carbohydrate content, which includes the dietary fibre, varied between 38-69%, as shown in Table 1. These preliminary results show that the analysed macroalgae can be interesting and alternative sources of protein, minerals, and, eventually, fibre.

4. CONCLUSION

In western countries, the use of seaweeds for human consumption is not part of traditional food habits. With globalization its consumption has been increasing. Moreover, deficient nutrition, mainly in underdeveloped countries, drives research on cheaper plant sources, with balanced macronutrients profile and with potential health benefits.

The results of this study show that the analysed macroalgae can be interesting alternative protein (especially *Porphyra tenera*) and minerals sources (essentially *Laminaria japonica)*, and, eventually, also of fibre. All the samples are poor sources of fat.

Further studies are being conducted in order to better characterize these and other species in what concerns their chemical composition and potential health effects.

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Caracterização do perfil de aminoácidos da carne do peito de faisão (*Phasanius colchicus*)

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Palavras chave: *Phasanius colchicus*; faisão; aminoácidos; proteína; HPLC/fluorescência.

RESUMO

Neste trabalho caracterizou-se o perfil de aminoácidos da carne do peito de faisão (n=10), liofilizada, proveniente de animais criados em regime semi-estensivo.
A quantificação do teor de proteína das amostras foi efetuada pelo método de Kjeldahl e o teor de aminoácidos avaliado por HPLC com detecção por fluorescência.
Os dados obtidos no presente estudo revelam que o perfil de aminoácidos destes animais, criados em regime semi-extensivo, é semelhante ao dos faisões selvagens. Assim, a sua criação neste regime permite a manutenção das suas características naturais, e contribui para o bem-estar dos animais e para a sustentabilidade do ecossistema.

1. INTRODUÇÃO

O faisão (*Phasaniu* sp.) diferencia-se em ecótipos, nomeadamente ocidental e oriental, que se distinguem, por sua vez, pela cor da região dorso-caudal do animal. O faisão comum (*Phasanius colchicus* L.), pertencente ao ecótipo ocidental, tem interesse para criação industrial e para a caça [1]. A criação industrial de faisões é realizada não só para repovoamento ou para largadas de caça, mas também para a produção de animais para consumo.
A carne de faisão, à semelhança de outras carnes brancas, é rica em proteínas e pobre em gordura, revelando um perfil nutricional interessante para a alimentação humana. Para além das qualidades nutricionais, esta carne é igualmente reconhecida pelas suas propriedades organopléticas, nomeadamente, por ser tenra e suculenta. No entanto, a qualidade da carne é influenciada pelas condições de crescimento e alimentação a que a ave foi sujeita [2].
Há estudos que mostram que, comparativamente com animais selvagens, a carne de faisão criado em cativeiro apresenta um teor proteico superior, uma vez que os animais estão sujeito a uma alimentação mais controlada, capaz de compensar as oscilações nutricionais do *habitat* selvagem [3].

A produção de aves cinegéticas em regime semi-extensivo tem despertado grande interesse por parte dos produtores que, desta forma, proporcionam aos animais não só um ambiente de bem-estar e liberdade, mas também uma resposta adequada às suas necessidades nutricionais. Neste trabalho, pretendeu-se caracterizar o perfil de aminoácidos da carne do peito de faisão criado em Portugal neste tipo de sistema (semi-extensivo), uma vez que estes dados não estão descritos.

2. MATERIAIS E MÉTODOS

2.1. Reagentes e padrões

L-alanina, L-arginina, L-asparagina, ácido L-aspártico, ácido L-glutâmico, L-cisteína, L-glicina, L-glutamina, L-histidina, L-isoleucina, L-leucina, L-lisina, L-metionina, L-fenilalanina, L-ornitina, L-prolina, L-serina, L-treonina, L-triptofano, L-tirosina, L-valina e L-norvalina (padão interno) e cloreto de dansilo (reagente de derivatização) foram obtidos da Sigma-Aldrich (St. Louis, MO, EUA). A água desionisada obteve-se a partir de um sistema de purificação de água Milli-Q (Millipore, Bedford, MA, EUA). Utilizou-se acetonitrilo de grau HPLC da Fluka (Madrid, Espanha). Todos os outros reagentes usados eram de grau analítico.
Para a identificação e quantificação dos compostos individuais preparou-se uma solução padrão com uma mistura de aminoácidos de 500 μg/ml. A partir desta, prepararam-se diluições com concentrações decrescentes (100 μg/ml; 50 μg/ml; 10 μg/ml; 5 μg/ml; 1 μg/ml; 0,5 μg/ml; 0,1 μg/ml).

2.2. Amostras

Os animais utilizados no estudo (n=10) foram criados em condições semi-extensivas na zona de Tomar (empresa produtora: Caça Brava). O ambiente natural da espécie foi recriado, com a presença de sorgo, árvores e arbustos.

2.3. Preparação das amostras

Após o abate, procedeu-se à remoção de penas, dissecação da carcaça e remoção dos músculos do peito. As amostras resultantes de cada animal foram individualmente trituradas e homogeneizadas numa picadora (Moulinex Classical A320R1, Moulinex, França), 3 x 5 segundos. Seguidamente, foram liofilizadas e armazenadas em tubos de polipropileno até serem analisadas.

2.4. Análises químicas

A quantificação do teor de proteína das amostras foi efetuada pelo método de Kjeldahl [4].
Para a determinação do perfil de aminoácidos, as amostras foram sujeitas a uma hidrólise ácida, seguida de derivatização com cloreto de dansilo, como descrito por Pimentel *et al.* [5]. A análise cromatográfica foi realizada num sistema integrado de HPLC Jasco (Jasco, Japão) equipado com injetor automático AS-950, bomba PU-980 e detetor de fluorescência FP-920

($\lambda_{excitação}$: 335 nm e $\lambda_{emissão}$:514 nm). A separação cromatográfica foi efetuada numa coluna de fase reversa Luna (5 µm; 150 x 4,60 mm) da Phenomenex (Torrance, CA, EUA), à temperatura controlada de 40 ºC. Os aminoácidos foram identificados por comparação do tempo de retenção de cada composto individual com o do respetivo padrão. A quantificação foi efetuada com base no método de padrão interno (com base na regressão da relação entre a resposta do analito e a resposta do padrão interno), utilizando os sinais de fluorescência. Os dados cromatográficos foram analisados utilizando o *software* Borwin-PDA (JMBS, França).

3. RESULTADOS E DISCUSSÃO

Os resultados podem ser observados na Tabela1 e Figura 1, onde são expressos em mg/g de carne (peso seco) e em mg/g proteína, respetivamente. Os resultados da Tabela 1 mostram que os aminoácidos essenciais predominantes nas amostras liofilizadas da carne do peito de faisão são a lisina e a leucina, seguidas da histidina, isoleucina, valina e metionina, da fenilalanina e da treonina. Quanto aos aminoácidos não essenciais, os teores mais elevados verificam-se na arginina, alanina e na soma dos ácidos aspártico e glutâmico, seguindo-se a tirosina, a glicina e, por último, a prolina.

Tabela 1. Perfil de aminoácidos da carne liofilizada de peito de faisão (mg aminoácido/g de carne p.s.).

Aminoácidos essenciais	mg/g carne p.s.	Aminoácidos não essenciais	mg/g carne p.s.
Lisina	103,7	Arginina	62,5
Leucina	62,7	Alanina	38,7
Histidina	58,1	Ác. Aspártico + Ác. Glutâmico	37,3
Isoleucina	35,1	Tirosina	27,3
Valina + Metionina	30,6	Glicina	22,8
Fenilalanina	27,6	Prolina	7,1
Treonina	24,3	Ornitina	n.d.
		Serina	n.d.

Os resuldados representam a média de 2 análises independentes.
Legenda: p.s. – peso seco; n.d. – não detetado.

Está descrito que a carne de faisão criado em aviário apresenta teores superiores de proteína, e consequentemente de aminoácidos, comparativamente com animais selvagens, fruto da influência do tipo de alimentação e ambiente a que os animais estão sujeitos [3].
Os dados obtidos no presente estudo, revelam que o perfil de aminoácidos destes animais criados em regime semi-extensivo é semelhante ao dos faisões selvagens. Assim, a sua criação em regime semi-extensivo permite a manutenção das suas características naturais, contribuindo para o bem-estar dos animais e para a sustentabilidade do ecossistema.

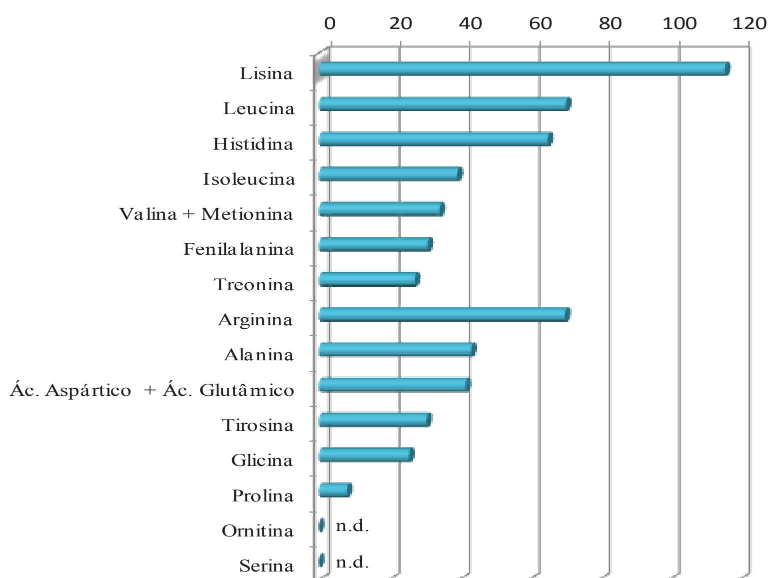

Figura 1. Perfil de aminoácidos da carne liofilizada de peito de faisão (mg aminoácido/g proteína). *Legenda: n.d. – não detetado.*

4. CONCLUSÃO

As aves cinegéticas, como é o caso do faisão, para além de suscitarem o interesse dos caçadores para atividades recreativas, são também usadas como uma fonte de proteína para consumo humano. A sua produção em regime semi-extensivo tem despertado grande interesse, uma vez que proporciona aos animais não só um ambiente de bem-estar e liberdade, mas também uma resposta adequada às suas necessidades nutricionais. Os resultados deste estudo mostram que a criação de faisão neste tipo de sistema contribui para a manutenção do seu perfil natural.

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Levels of antioxidants in simple and combined forms of fruits and vegetables: leaf lettuce (*Lactuca sativa* L., var. *crispa*) and cape gooseberry (*Physalis peruviana*)

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Keywords: Antioxidant capacity; synergism; mixtures; interaction

ABSTRAT

In this study, pure extracts and different mixtures of a fruit (cape gooseberry) and a vegetable (leaf lettuce) were analyzed to identify possible synergistic antioxidant combinations. The total phenolic compounds and the flavonoids content were determined, as well as two assays to evaluate the antioxidant activity: ferric reducing antioxidant power (FRAP) and DPPH$^{\bullet}$ scavenging activity.
Cape gooseberry was the sample with the highest antioxidant activity, also providing a higher content of phenolic compounds and flavonoids. Accordingly, within the mixtures, the best results were obtained with lettuce/cape gooseberry mixture (25%:75%).
The referred combination (25%:75%) was selected as the most interesting mixture to explore the occurrence of possible synergistic and antagonistic interactions in future studies.

1. INTRODUCTION

According to the World Health Organization guidelines people should consume 400-600 g per day of fruits and vegetables to achieve the amount of fiber and phytochemicals (including polyphenols) to reduce the risk of several chronic diseases. However, most of the European people do not ingest sufficient amounts of these products. The changes driven by modern society had impact not only on style and quality of life, but also brought to consumers an increasing conscious of health and wellbeing [1,2].
Oxidative damage plays an important role in the development of several chronic diseases, including diabetes, cardiovascular dysfunctions, and cancer. Despite the human body have a well sophisticated antioxidant defense system, many environmental and life style factors could introduce an imbalance between antioxidant defense and free radical damage [3].
The phenolic compounds of fruits and vegetables comprise the ability to neutralize active oxygen species and free radicals and, therefore, can play an important role in the prevention of degenerative diseases [4].
The aim of this study was to combine a fruit and a vegetable in different proportions in order to achieve a greater antioxidant profile. Lettuce (*Lactuca sativa* L., var. *crispa*) is a very

common leafy vegetable mainly consumed fresh and used in a normal healthy dietary pattern [5]. On the other hand cape gooseberry fruit (*Physalis peruviana*) is a berry with a fleshy pulp with numerous seeds enclosed by a calyx. It is now widely consumed in subtropical areas, being important to know its bioactive composition [6]. The combination of this two food matrices could lead to a synergistic or antagonistic effect due to the interaction of the antioxidants present in each one.

2. MATERIAL AND METHODS

2.1. Samples collection and preparation

Fresh lettuce leaves (*Lactuca sativa* L., var. *crispa)* were selected in local supermarkets and cape gooseberry *(Physalis peruviana)* was obtained from a domestic producer.
Briefly, lettuce leaves and cape gooseberry were triturated separately and then combined in 5 different lettuce/cape gooseberry proportions: 100%:0%, 75%:25%, 50%:50%, 25%:75% and 0%:100%. Water was added to each sample till a 40 ml volume was achieved. After magnetic stirring during 30 minutes, extracts were filtered through a Whatman No. 4 paper filter. The extraction process was performed in quadruplicate. The extracts were used to perform the assays.

2.2. Chemical analyses

The total amount of flavonoids and phenolic compounds was determined spectrophotometrically using a microplate reader Synergy^{TM} HT (Bio-Tek Instruments, Inc) based on Costa *et al.* [7] and Alves *et al.* [8], with some modifications.
Antioxidant capacity was determined using two assays, DPPH$^{\bullet}$ inhibition [9] and ferric reducing antioxidant power (FRAP) assay [10], with some modifications.

3. RESULTS AND DISCUSSION

The results of the different lettuce/cape gooseberry mixtures evaluated are presented in Table 1.
The pure cape gooseberry (100%) presented the highest content of the bioactive compounds analyzed and the highest antioxidant activity expressed as FRAP. If the difference between lettuce and cape gooseberry in what concerns to flavonoids content is great (2.8 mg EE/100 g in lettuce and 36.9 mg EE/100 g in cape gooseberry) the same is not true with the total phenol contents (19.1 mg EE/100 g and 28.3 mg EE/100 g) presenting more similar values. Lettuce is an important source of phenolic compounds that contributes to the antioxidant activity of the vegetable. Still, it is known that the concentration of phenolic acids and flavonoids are sensitive to environmental conditions, post-harvest processing and storage which could result in significant losses in bioactive compounds [11,12].

Results presented showed that cape gooseberry present higher contents of bioactive compounds associated with higher antioxidant activity than lettuce. Indeed, in mixtures an increasing content of these compounds were observed as the proportion of the fruit increase. However, the different mixtures of the two food matrices have lower flavonoids content than expected, seeming to occur an antagonistic effect in what concerns this parameter. A similar behavior can be described for total phenolic values determined in the different combinations. Taking into account the FRAP values, an antagonistic behavior can be considered, once the values determined were always lower than expected.

In the case of the DPPH$^{\bullet}$ inhibition assay, the value obtained with 100% cape gooseberry extract is two fold higher than the determined with lettuce (40.5 and 19.7%, respectively). Nevertheless, the best behavior was obtained with the combination 25% lettuce and 75% cape gooseberry. The mixture 50%:50% have also a similar value, near 40%. It seems clear the synergistic effect obtained with the addition of cape gooseberry to lettuce, taking into account this parameter.

Table 1. Antioxidant activity, phenolic and flavonoid contents of different lettuce/cape gooseberry combinations.

Samples		**Antioxidant activity**		**Polyphenols**	
		FRAP mg FSE/100 g	DPPH % inhibition	Total phenolics mg GAE/100 g	Flavonoids mg EE/100 g
Lettuce	**Cape gooseberry**				
100 %	0%	165.77 ± 0.36	19.71 ± 0.42	19.09 ± 0.17	2.80 ± 0.07
75%	25%	97.14 ± 1.36	29.03 ± 1.07	11.24 ± 0.24	4.26 ± 0.13
50%	50%	138.31 ± 1.13	39.27 ± 1.17	11.83 ± 0.13	6.03 ± 0.22
25%	75%	197.20 ± 2.79	44.84 ± 0.86	14.67 ± 0.41	9.48 ± 0.43
0%	100%	296.49 ± 1.92	40.47 ± 1.89	28.25 ± 0.35	36.92 ± 0.32

Results are reported as mean ± SD.

FSE (Ferrous sulfate equivalents); GAE (Gallic acid equivalents); EE (Epicatechin equivalents).

Antioxidants are present in vegetables and fruits in different amounts and combinations. If these specific foods are consumed together the total antioxidant capacity may be modified by via synergistic, additive or antagonistic effects, result of the interactions between different phytochemicals [3]. Combined effects of antioxidants could be superior to their individual effects [13]. Moreover, studying different types of interactions within and across different food groups is important to identify the best antioxidant profile of a mixture in order to achieve the maximum antioxidant potential of food. Wang *et al.* [3] showed that combining specific foods of different food categories (fruit, legumes and vegetables) was more likely to result in synergistic antioxidant capacity that combination within the same food group. The results of this work are not totally in accordance with the referred authors. However, it should be noted that, according to same authors the number of antagonisms identified was approximately equal to the number of synergisms within or across food groups.

The possible synergetic/antagonistic effect found in this work will be explored with further studies. Nevertheless, antagonistic effects must also be identified since they may have negative effects in human health.

4. CONCLUSION

Cape gooseberry pure extract (100%) presented the highest antioxidant activity by FRAP assay and also the highest content of phenolic and flavonoids content. Among the different combinations analyzed the mixture lettuce 25% and cape gooseberry 75% seems to have the best antioxidant activity as well the combination lettuce 50% and cape gooseberry 50%. Further studies will be done in order to explore the possible synergistic/antagonistic effect and strategically select appropriate food combinations for achieve synergistic effects.

Acknowledgments

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Tocopherols in rainbow trout (*Oncorhynchus mykiss*) muscle: optimization and validation of an extraction method

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Keywords: vitamin E; *Gracilaria vermiculophylla*, method development

ABSTRACT

In this work, five different methods of extraction to quantify vitamin E in the muscle of rainbow trout were compared. Solid-liquid extraction with *n*-hexane provided the best results showing high correlation coefficients (> 0.999), high precision (< 3.0%) and good recoveries (>96%). This extraction method was then optimized and validated. The detection and quantification limits were 40 and 121 ng/g of fish muscle, for α-tocopherol, and 111 ng/g and 338 ng/g, for γ-tocopherol, respectively.

The method was used to quantify tocopherols in the muscle of rainbow trout fed two different diets: a fishmeal-based (FM) diet (control) and a diet containing 5% of *Gracilaria vermiculophylla* meal to replace FM . Only α-tocopherol and γ-tocopherol were found in the samples. A reduction of tocopherols content in the flesh of the group fed seaweed-based diet was observed.

1. INTRODUCTION

Rainbow trout (*Oncorhynchus mykiss*) exhibits a valuable content in long-chain n-3 polyunsaturated fatty acids (PUFAs), which are well known for their health benefits [1]. In order to protect PUFAs from lipid oxidation, preservation methods such as modified atmosphere packaging or freezing are commonly used. However, they do not completely hinder lipid oxidation, which is responsible for quality deterioration of fish and development of unpleasant flavors and odors. In aquaculture, the use of dietary antioxidants such vitamin E may minimize the formation of peroxyl radicals during fish storage [2].

In fish, α-tocopherol is the main form of vitamin E found and its deposition in flesh depends on the ingredients and/or synthetic forms of vitamin E included in aquafeeds [3]. Seaweeds have become an important ingredient in fishfeed due to their content in bioactive compounds. Nevertheless, no data exist evaluating the effects of dietary seaweed inclusion on vitamin E content of fish muscle. In this work, an extraction method for tocopherols quantification in

rainbow trout muscle was developed and validated. Then, it was applied to muscle samples of rainbow trouts fed two different diets: a fishmeal-based diet (control) and a diet containing 5% of a red macroalgae.

2. MATERIAL AND METHODS

2.1. Samples

A control diet (CTRL) was formulated according to nutritional requirements of rainbow trout (*O. mykiss*). In the seaweed diet (G5), fish meal was adequately replaced by 5% of *Gracilaria vermiculophylla* meal. The remaining ingredients were adjusted in order to answer to all nutritional requirements of the animals. Each group was fed for 91 days. After that, animals were sacrificed and muscle samples collected, frozen in liquid N and stored at -80ºC till analysis.

2.1. Sample extraction

Samples (~1 g) were spiked with 10 µl of tocol (1 mg/ml), and extracted with 5 ml of absolute ethanol and 10 ml of *n*-hexane (0.01% of BHT). After vortexing and constant agitation (30 min), 1% of NaCl solution was added. After centrifugation, the supernatant was collected and the residue re-extracted twice with *n*-hexane. Organic layers were combined and Na_2SO_4 was added. After a new centrifugation, the supernatant was collected and evaporated under a N stream. The residue was, then, re-suspended with 500 µl of *n*-hexane and filtered before injection into the HPLC system.

2.2. Chromatographic analysis

Chromatographic analysis was performed in a HPLC-DAD/fluorescence system (Jasco, Japan). Compounds separation was achieved using a Supelcosil™ LC-SI (3 µm, 75x3 mm) column (Supelco) and *n*-hexane/1,4-dioxane (98:2) as eluent, at a flow rate of 0.7 ml/min. Tocopherols were identified by comparison with standards and by their UV spectral characteristics. Quantification was performed on the basis of the internal standard method using the fluorescence signals (λ_{exc}:290nm; λ_{em}:330nm).

3. RESULTS AND DISCUSSION

In order to select the best extraction method to extract vitamin E in the experimental samples, 5 different procedures frequently applied to foodstuffs were tested in rainbow trout muscle samples (Table 1). Only α-tocopherol and γ-tocopherol were found in the muscle. The best extraction rates for α-tocopherol were achieved with method C, but γ-tocopherol was found below the detection limit. Method A extracted several interfering substances which impaired vitamers identification. The other methods (B, D, E) displayed significantly lower α-tocopherol recoveries ($p<0.05$). Although saponification is a common step for vitamin E extraction in salmonids flesh [4], its application before *n*-hexane extraction (E) did not

contributed to a more effective extraction of tocopherol content when compared to method C. In that case, tocol had to be added after KOH neutralization since it is degraded during saponification. Method C was then optimized in order to reduce time and reagents consumption, and increase the yield of extraction of the compounds. Different combinations of solvents were tested (Table 2). Moreover, the effect of reducing extraction time (15 and 5 min) was investigated, but results were not promising since some compounds extraction was impaired. The method which provided the best results is entirely described in Materials and Methods section. It showed to be accurate (mean recoveries: 97±2% and 104±5%, for α- and γ-tocopherol, respectively), presented high correlation coefficients (>0.999) and intra- and inter-day precisions (<3.0 %). Limits of detection were 40 and 111 ng/g of fish muscle, for α- and γ-tocopherol, respectively, and limits of quantification were 121 ng/g (for α-tocopherol) and 338 ng/g (for γ-tocopherol).

Table 1. Vitamin E concentration expressed in mg/kg of muscle in fresh weight basis (*n*=4).

Method	Reference	Vitamin E	
		α-tocopherol	γ-tocopherol
A) Soxhlet extration	[5]		
B) Folch method	[6]	11.76 ± 0.46^{d}	< LD
C) Solid-liquid *n*-hexane extraction	[7]	16.11 ± 0.42^{a}	< LD
D) Solid-liquid methanol extraction	[8]	13.56 ± 0.36^{c}	NF
E) Saponificaton followed by n-hexane extraction	[4]	15.09 ± 0.34^{b}	< LD

Different superscript letters indicate significant differences between methods ($p<0.05$).

Table 2. Relative areas (%) of α- and γ-tocopherol under different experimental conditions.

Experimental conditions	Relative area (%)	
	α-tocopherol	γ-tocopherol
Solvent amount		
5 ml absolute ethanol + 10 ml *n*-hexane	100	100
2.5 ml absolute ethanol + 10 ml *n*-hexane	97	83
2.5 ml absolute ethanol + 5 ml *n*-hexane	96	83
10 ml *n*-hexane: ethyl acetate (90:10)	54	51

The final validated method was used to analyse the muscle from rainbow trout fed two different diets. Fish fed G5 showed lower levels of α- and γ-tocopherol in the muscle (14.74 and 0.69 mg/kg, respectively), comparatively with control (18.34 and 1.01 mg/kg, correspondingly).

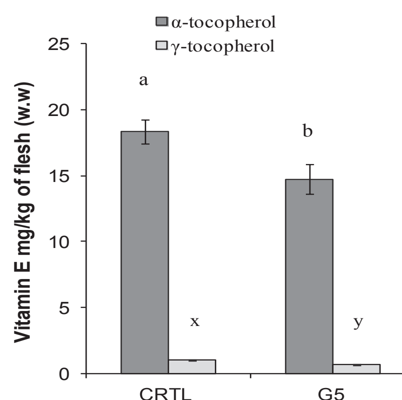

Figure 1. Vitamin E content in the muscle of rainbow trout fed experimental diets. Values are expressed as mean ± S.D. (*n*=12). Different superscript letters indicate significant differences between methods ($p<0.05$).

Although *G. vermiculophylla* is described as a good source of protein and several bioactive compounds, including α-tocopherol (86.5 mg/kg lipids) [9], the readjustment of the diet ingredients to include 5% of *Gracilaria* seems to provide lower tocopherol amount, which was probably reflected in the fish muscle content.

4. CONCLUSIONS

In this study, different methods of extraction to quantify vitamin E in the muscle of rainbow trout were compared. Solid-liquid extraction with *n*-hexane provided the best results. The extraction method was then optimized and validated.
As expected, α-tocopherol was the main compound found in muscle samples. Another vitamer (γ-tocopherol) was also found in lower amounts. Muscle samples of rainbow trout fed with commercial and experimental diets were analysed. A reduction of vitamin E in the flesh of the group fed seaweed diet (in which fish meal was replaced by 5% of *Gracilaria* meal) was observed.

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DNA barcoding coupled to high resolution melting analysis for saffron (*Crocus sativus* L.) authentication

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Keywords: saffron; authenticity; PCR; barcoding

ABSTRACT

Saffron is the most expensive spice in the world, which is obtained from the red-dried stigmas of the flowered *Crocus sativus* L. As a consequence of its high market value, saffron has been frequently associated with an unrivaled degree of adulteration. Recently, molecular biology techniques using DNA barcodes for species identification has gained worldwide attention, with potential for recognition of animals, plants and fungi. The internal transcribed spacer (ITS) of nuclear ribosomal DNA and the portions of the plastid *matK* gene have been most frequently proposed as potential DNA barcodes for the identification of plant species.,

The aim of this work was to develop molecular markers to evaluate the authenticity of saffron. DNA was extracted from different leave samples of *C. sativus* L. and other *Crocus* spp. by the CTAB-based method. All extracts were amplified by polymerase chain reaction (PCR) targeting three different DNA barcode loci, namely ITS1, ITS2 and *matK* and a SCAR marker. A real-time PCR assay using EvaGreen dye combined with High Resolution Melting analysis was carried out to discriminate different species of *Crocus* genus, being further confirmed by sequencing. The primers targeting ITS1 and *matK* loci were specific for the genus *Crocus*, while the primers targeting ITS2 locus and the SCAR marker were specific for the species *C. sativus* L. This study revealed that genomic tools and, specifically, DNA barcoding can provide easy, fast and reliable methods for saffron authentication.

1. INTRODUCTION

Saffron is used for flavoring and coloring in food preparation as well as in traditional and modern medicine. Owing to its high market value, saffron has been frequently related to an unrivaled degree of adulteration performed in both grounded and whole stigma with the most diverse materials and strategies. Thus, the detection of adulterants becomes a very important issue for the evaluation of product value, to ascertain the unfair competition and to assure consumer protection against fraudulent practices. So far, different methods based on chemical and sensorial parameters have been developed to assess the quality and authenticity of saffron. However, saffron authentication relies mostly on microscopic observation of morphological traits, which is time-consuming, depends on experienced personal personnel and it is susceptible of subjective interpretation [1,2].

Nowadays, DNA-based methods have proved to be excellent tools for the analysis of highly processed foods [3], spices [2] and [4]. The majority of these methods are based on PCR coupled with specific markers (like SCAR) [5] or with the recent approach of DNA barcoding to identify a particular species [6]. In this new approach targeting small DNA regions, it is possible to distinguish closely related species. The high variability of ITS and the high resolution of the *matK* gene are the main reasons for their widespread use as DNA barcode in plants.

The main objective of this study was to develop molecular markers, with special emphasis on DNA barcoding, to evaluate the authenticity of saffron. For species identification, the adequacy of three *loci* (ITS1, ITS2 and *matK*) and a SCAR marker was tested using real-time PCR with high resolution melting (HRM) analysis, as a simple and fast tool to authenticate saffron.

2. MATERIALS AND METHODS

2.1. Sampling and DNA extraction

A total of fourteen lyophilized leave samples of *C. sativus* L. and other *Crocus* spp. were acquired, from which some were provided by the Bank of Plant Germplasm of Cuenca, others were sent from Arabia Saudita or collected in Cinfães (North region of Portugal). Eight commercial samples of *C. sativus* L. stigmas and powder were acquired at the retail market. *C. sativus* L. and *C. cartwrightianus* bulbs were also used. To test the specificity of the developed method, 18 samples of different condiments were tested. All samples were adequately stored prior to DNA extraction. DNA extraction was performed using the CTAB method as described by Mafra et al. [7] with minor adaptation (lysis buffer with polyvinylpyrrolidone and β-mercaptoetanol).

2.2. Qualitative PCR and Real-time PCR

Qualitative PCR was carried out in 25 µL of total reaction volume containing 2 µL of DNA extract, 67 mM of Tris-HCl (pH 8.8), 16 mM of $(NH_4)_2SO_4$, 0.01% of Tween 20, 200 µM of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Germany), 3.0 mM of $MgCl_2$ and 300 nM of each primer (Table 1). The reactions were performed in a thermal cycler MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, USA) with a set of temperature specific for each primer.

The amplifications by real-time PCR were carried out in 20 µL of total reaction volume containing 2 µL of DNA extract, 1x of SsoFast™ Evagreen® Supermix (Bio-Rad Laboratories, USA) and 250 nM of each primer targeting the locus ITS1 (Table 1). The real-time PCR assays were performed on a thermal cycler CFX96 Real-Time System (Bio-Rad Laboratories, USA) using the following conditions: 95ºC for 5 min; 45 cycles at 95ºC for 30 s followed by 65 C for 60 s, with collection of fluorescence signal at the end of each cycle. Data were collected and processed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad

Laboratories, USA). The fluorescence data were processed using the Precision Melt Analysis 1.2 (Bio-Rad Laboratories, USA) in order to discriminate different species of *Crocus* genus.

2.3. Sequencing of ITS1 region

To complement real-time PCR data, ITS1 region of DNA of some samples were sequenced. Firstly, all samples were amplified according to the optimised conditions and in 50 µL of total reaction volume. All PCR products were purified and sent to StabVida (Caparica, Portugal) for sequencing. The sequencing data were analysed using the available software BioEdit v7.2.5 (Ibis Biosciences, Carlsbad, CA, USA) and FinchTV (Geospiza, Seattle, WA, USA).

Table 1 – Oligonucleotide primers used in this study.

Primers	Sequence (5'-3')	Target	Amplicon size (bp)
ITS-CS-F **ITS-CS-R1**	GATCGCGAACGTGTTACACCAT TATATCCACGCCTGCGTCCCT	ITS1	226
ITS-CS-F **ITS-CS-R2**	TCGATTCGTCGGTTACCATCACAC TCTTTGAAGGACCATAGGGTTCCA	ITS2	641
MKCS-F **MKCS-R**	GATCGCGAACGTGTTACACCAT TCTGATTCCGAGGACGGTTC	*matK*	606
CS-F **CS-R**	CTACGTTCCAGTTCGCTGTTTG GTGGTGTCGCTGGTAGACTGAAA	SCAR marker	274

3. RESULTS AND DISCUSSION

The assay specificity was tested using different plant species to evaluate any possible cross-reactivity. From the 18 different condiments investigated, no cross-reactivity was observed, confirming the adequate specificity of the assay for *Crocus* spp identification. The DNA extracted from each sample was amplified with the four set of primers enabling to obtain PCR fragments of 226 bp, 641 bp, 606 bp and 274 bp for the three genomic regions of ITS1, ITS2, *matK* and the SCAR marker, respectively. The results also showed that the loci ITS1 and *matK* were specific for *Crocus* genus, while the SCAR marker and ITS2 loci were specific for the species *Crocus sativus* L. All these markers were considered suitable for studies of authenticity and species identification in all the tested plant parts, with the exception of the ITS2 region whose amplification was not possible using the stigmas.

The real-time PCR combined with HRM analysis was applied to the ITS1 in order to distinguish the different *Crocus* species within the same assay. This was achieved since the HRM analysis was able to separate samples belonging to distinct species in different groups with a high level of confidence, only based on small nucleotide differences present in the sequence (Figure 1). Thus, this approach also proved to be useful in species identification and to distinguish closely related species.

To confirm HRM results and to verify the nucleotide differences on the basis of the separation of species in different groups, some stigmas, bulbs and leaves samples of *Crocus* spp. were sequenced. The results showed that all samples of the *Crocus sativus* L. species presented the

same sequence. When compared to other species from the same genus, the expected sequence of *Crocus sativus* L. had some nucleotide differences with all the other tested species of *Crocus* genus. In addition, distinct species from *Crocus* genus also presented nucleotide variations among them and with the targeted sequence of *Crocus sativus* L. The results also revealed, with a high reliability, the existence of some errors in the available sequences in the NCBI database. The ITS1 locus revealed to be a promising tool for distinguishing different species of the genus *Crocus*.

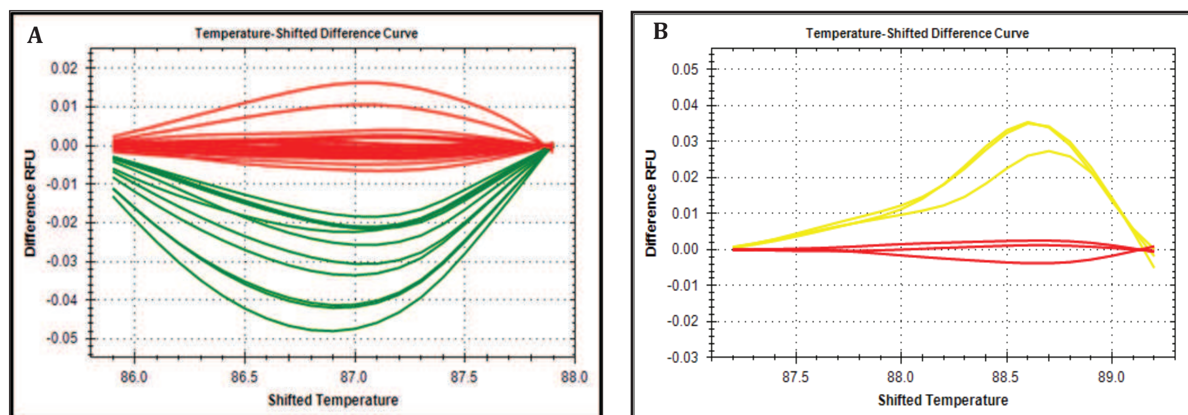

Figure 1. HRM analysis of *Crocus* species with primers ITS-CS-F/ITS-CS-R1. Difference curves obtained by HRM analysis of leave samples (A); difference curves obtained by HRM analysis of bulbs samples (B). Red curves indicate *Crocus sativus* L. species, green and yellow curves indicate other *Crocus* spp.

4.CONCLUSION

This study shows the high potentialities of the combination of DNA barcode with HRM analysis to successfully identify and distinguish closely related species. It also reveals the high reliability of DNA-based methods in the authenticity assessment in order to detect fraudulent practices, so common in saffron and other spices and herbs.

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Antioxidant activity and cytotoxic effects of polar extracts from saffron (*Crocus sativus* L.) flowers

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Palavras chave: *Crocus sativus*; antioxidant activity; citotoxicity

ABSTRACT

Saffron is cultivated exclusively to obtain the stigma of its flowers, which represents less than 10% of the flower weight. In fact, to achieve a single kg of dried stigma, more than 60 kg of floral bio-residues are produced. These bio-residues were reported as having high bioactivity and important contents in bioactive compounds. Before considering the use of these bio-residues in different applications, it is important to assess its cytotoxicity. Herein, the antioxidant activity and cytotoxicity of saffron flower polar extracts were evaluated. All samples showed antioxidant activity, despite the higher effectiveness of the hydroalcoholic extract. Up to the concentrations corresponding to the EC_{50} values obtained in the antioxidant activity assays, none of the extracts showed high cytotoxicity against Caco-2 cell lines. Accordingly, saffron flowers might be used in different applications such as the development of food supplements or pharmaceutical related products.

1. INTRODUCTION

Saffron (*Crocus sativus* L.) flower is composed of six purple tepals, three yellow stamens and a white filiform style ending in a stigma with three threads, which represents less than 10% (w/w) of the flower weight. Nevertheless, saffron is cultivated for the stigma, which, after being dried, is the most valued spice [1]. For each kg of this spice, about 63 kg of floral bio-residues are produced, which so far are not exploited, being usually thrown away. The floral bio-residues were reported as having high phenolic content and bioactive properties, justifying the study of its cytotoxic effects [2]. Furthermore, the effectiveness of bioactive compounds extraction from plants, as well as their corresponding activity, is highly dependent on factors such as different types of solvent, solvent-to-solid ratios and specially the solvent polarity [3]. Accordingly, different polar extracts from saffron flower were prepared and their antioxidant activity and cytotoxicity were compared.

2. MATERIALS AND METHODS

Different polar extracts (ethanol, ethanol:water 1:1 v/v, and water) were prepared using a solid to solvent ratio of 1:30. The antioxidant activity was evaluated by: 1) 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH discoloration using the formula: $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. 2) ferric reducing antioxidant power (FRAP), performed by mixing diluted extracts with the FRAP solution. Absorbance was measured at 595 nm. A calibration curve was prepared with ferrous sulfate and FRAP was expressed as mg of ferrous sulphate equivalents (FSE)/L of extract. 3) inhibition of β-carotene bleaching assay, evaluated through the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, measured as: β-carotene absorbance after 2h of assay/initial absorbance) × 100%. The cytotoxicity of extracts was evaluated in Caco-2 (ATCC® HTB-37™) cultures by using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) [4]. Caco-2 was cultured in DMEM medium supplemented with 10% heat-inactivated foetal bovine serum. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 ºC. To carry out the experiments, 10 000 cells were seeded in 96-well plates (flat bottom) and allowed to adhere overnight at 37 ºC. For cell treatment, these were incubated at 37 ºC for 1h and 24h in the presence of a variable number of extract concentrations in addition to a negative (medium without extracts) and positive control (Triton X-100, 1%). Absorbance was measured at 570 nm using a Cambrex ELX808 microplate reader (Biotek, Instruments, Inc; Winooski, VT, USA).

3. RESULTS AND DISCUSSION

3.1. Antioxidant activity

All samples proved to have antioxidant activity, despite the higher effectiveness of the hydroalcoholic extract. The antioxidant activity showed a concentration-dependent behavior, as it can be deduced from **Figures 1a-b**. The higher activity shown by hydroalcoholic extract is also reflected by the obtained EC_{50} values - DPPH scavenging activity, aqueous: 2.8±0.1 mg/mL; ethanolic: 2.4±0.1 mg/mL; hydroalcoholic: 1.5±0.1 mg/mL; β-carotene bleaching inhibition, aqueous: 2.7±0.1 mg/mL; ethanolic: 1.4±0.1 mg/mL; hydroalcoholic: 1.0±0.1 mg/mL. Likewise, the hydroalcoholic extract showed the highest FRAP: aqueous: 0.85±0.02 mM FSE/g dw; ethanolic: 1.02±0.03 mM FSE/g dw; hydroalcoholic: 1.32±0.02 mM FSE/g dw.

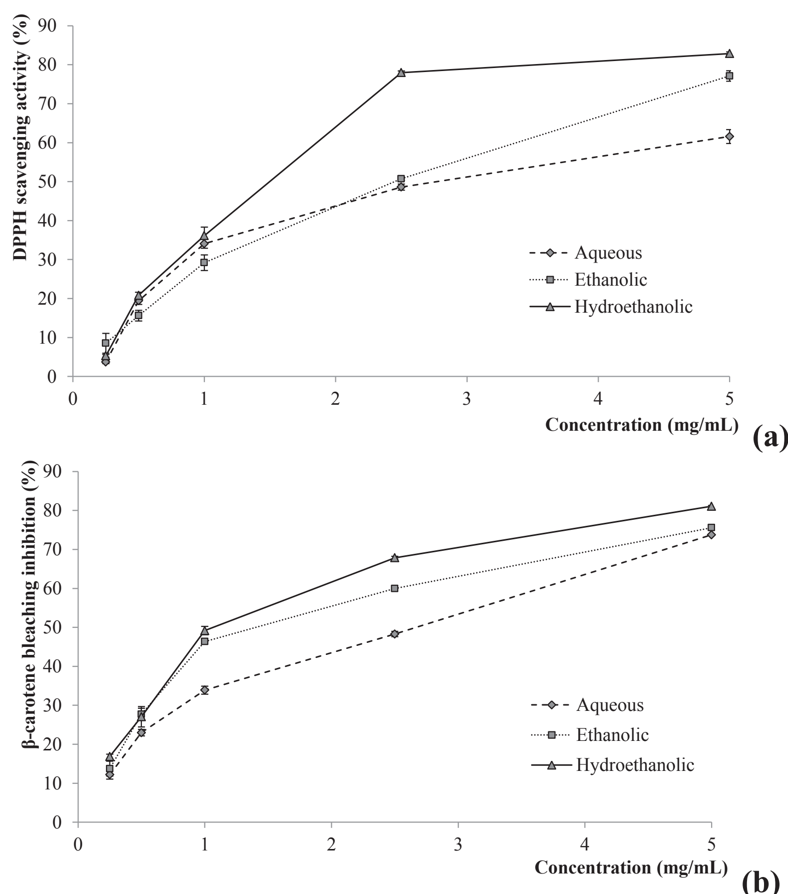
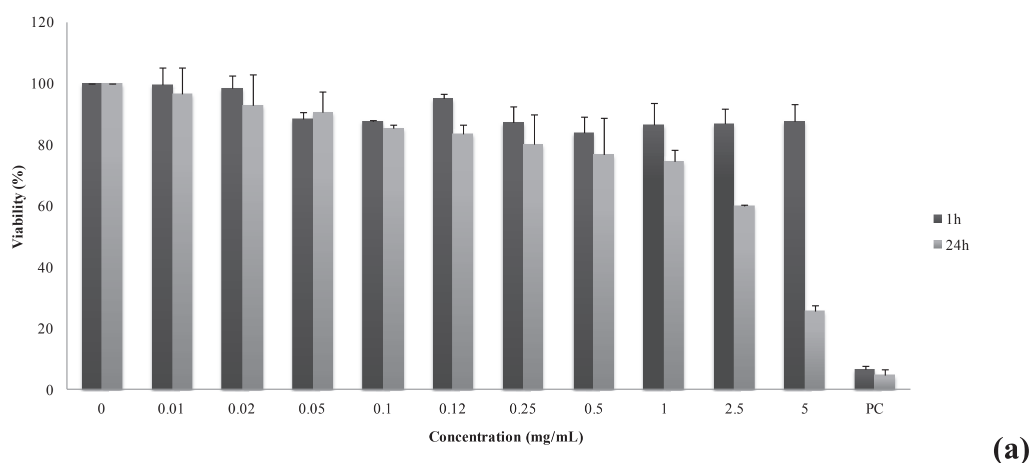


Figure 1. DPPH scavenging activity (a) and β-carotene bleaching inhibition (b).

3.2. Cytotoxicity

Results obtained from MTT assay are represented in **Figure 2a-c**. Saffron extracts induced dose-dependent decreases in viability percentages of Caco-2 cells, particularly pronounced at 24 h treatments (but also observable after 1h of exposure to hydroethanolic and ethanolic extracts). The comparison of the different tested extracts shows that the aqueous one is the less toxic to Caco-2 cells as a decrease in cell viability is observed only after 24h of treatment. The cytotoxicity observed herein is in agreement with previous studies [5], despite being lower when compared to the reported for different cell cultures [6].



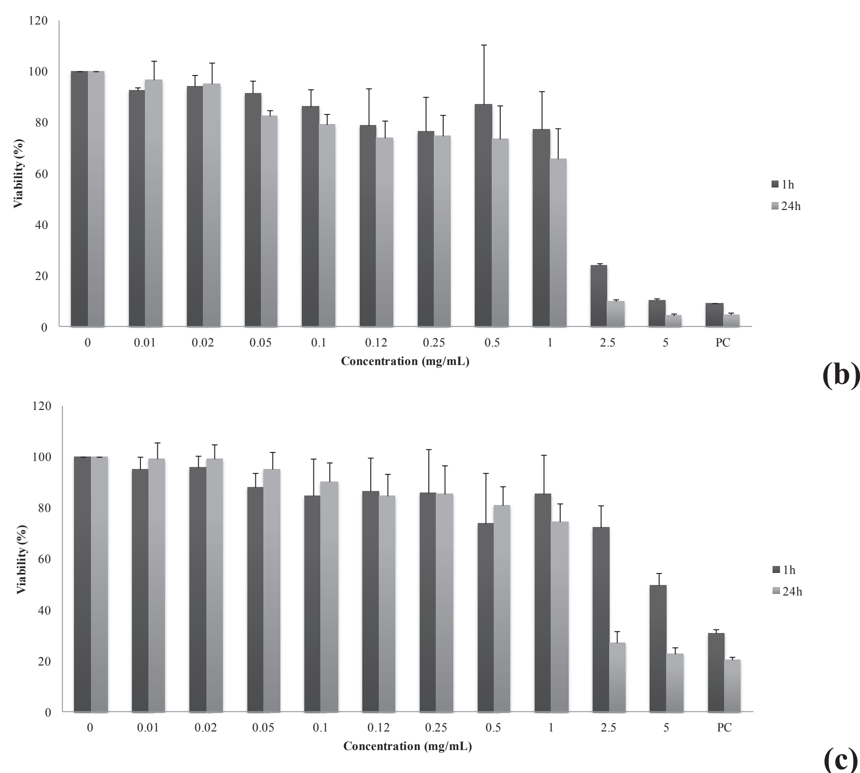


Figure 2. Viability of Caco-2 cells treated with saffron extracts. Results from MTT assay testing (a) aqueous extract, (b) hydroethanolic extract, and (c) ethanolic extract.

4. CONCLUSIONS

The main cytotoxic effects were only observed in the concentrations above the antioxidant activity EC_{50} values. The bioactivity and cytotoxicity were clearly influenced by the solvent polarity. Accordingly, saffron flowers might be used in different applications such as the development of food supplements or pharmaceutical related products.

Acknowledgments

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Evaluation of the antioxidant potential of *Castanea sativa* by-products: shell and bur

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KEYWORDS: *Castanea sativa* Mill; by-products; sustainability; antioxidant activity; polyphenols

ABSTRACT

Chestnut (*Castanea sativa* Mill.) is an abundant seasonal fruit in south Europe. During its processing, a large amount of waste material is generated. These agro-wastes can be valorized in order to achieve valuable phytochemicals and to improve the sustainability of the chain production. For that, chestnut by-products (shell and bur) collected from three Portuguese regions (Trás-os-Montes, Minho and Beira Alta) were studied to evaluate their antioxidant potential.

In order to achieve the best solvent to attain this goal, different extracts (aqueous, alcoholic and hydroalcoholic 1:1) were analyzed regarding the total polyphenols content in Minho shell. Hydroalcoholic extract was selected and the total phenolic content and DPPH scavenging activity evaluated for all samples.

The obtained results varied considerably between regions. Chestnut shell from Trás-os-Montes revealed the best values for total phenols (796.8 mg GAE/g db) and the lowest EC50 values for DPPH (318 µg Trolox /g db). Chestnut burs from Beira Alta presented the best results for total phenols (92.2 mg GAE/g db) and the highest EC50 values (0.8 µg Trolox/g db). The lowest EC50 values were obtained for Minho burs (0.4 µg Trolox/g db), and the same extract presented good values for the total polyphenols (85.3 GAE/g db). A reason for this variability could be the climatic conditions and the cultivars used in this production. The obtained results indicate a high potential of application for chestnut by-products, normally considered as disposable, in different industries such as pharmaceutical or cosmetic.

1. INTRODUCTION

Castanea sativa Mill. is a specie of Fagaceae family that can be found in south Europe and Asia (China). In Portugal chestnut trees are mainly used for nut production, representing a total area of about 35 000 ha with a production of about 19 000 t/year [1]. The nuts are consumed locally and exported, predominantly to Spain, Italy and France [2]. Trás-os-Montes is the major Portuguese region of chestnut production (over 75% of all production), being one of the most important economic resource in the region [1]. During its industrial processing, a large amount of waste material is generated: pericarp (outer shell), integument (inner shell)

and bur that surround the edible nuts. These chestnut by-products are being studied as extracts, revealing to be a good source of phenolic compounds with marked biological activity [3-4]. Vazquez *et al.* also studied chestnut shell extracts as heavy metal adsorbent [4] and its potential use as phenol substitutes in adhesives formulation and as chrome substitutes in leather tanning [5]. Improving the value of these wastes can provide a new sustainable way for chestnut processing companies. The aim of this paper was to evaluate chestnut shell and burs extracts from three different Portuguese regions and analyze their antioxidant potential, predicting their future applications in different industries, always considering sustainability and environmental protection.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Folin–Ciocalteu's reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH•) free radical, catechin, gallic acid, a water-soluble derivative of vitamin E (Trolox) were purchased from Sigma–Aldrich (Steinheim, Germany). Ethanol reagent grade was purchased from Fluka (Spain). Ultra-pure water was obtained using a Milli-Q water purification system (TGI Pure Water Systems, USA). Sodium acetate and sodium carbonate were purchased from Merck (Darmstadt, Germany).

2.2. Samples

Samples (shell and bur) from three portuguese regions (Minho, Beira Alta and Trás-os - Montes) were collected in September of 2013 and kept at - 20 ºC, protected from light, until analyze.

2.3. Extracts Preparation

Simulating the traditional use, the fruit with shell was previously cooked in an oven (Tecnogás, Portugal) at 240 ºC during 35 minutes. Samples were milled at particle size of approximately 0.2 mm using a basic mill (Grindomix Retsch, Düsseldorf, Germany) and stored in plastic tubes at 5 °C until analyze. Shell and bur samples (2.5 g) were submitted to solvent extraction by maceration with 20 mL of three different solvents: ethanol, ethanol: water (1:1) and distilled water for 30 min at 50ºC. Extracts were filtered through Whatman No. 1 filter paper, concentrated under vacuum at 37 °C or lyophilized, and kept under refrigeration (4 °C) prior to use.

2.4. DPPH radical scavenging activity

The antioxidant activity was evaluated by $DPPH^{\bullet}$ radical scavenging activity. The reaction mixture occurred in a 96 wells plate, and was constituted by the different samples concentrations (30 µL) and methanol solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm [6]. A calibration curve was prepared with Trolox (linearity range: 5–100 µg/mL, $R^2 > 0.9995$). The radical scavenging

activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: % RSA = [(ADPPH-AS)/ADPPH] × 100, where AS is the absorbance of the solution when the sample extract has been added at a particular level and ADPPH is the absorbance of the DPPH solution. The extract concentration providing 50% of radicals scavenging activity (EC50) was calculated from the graph of RSA percentage against extract concentration. BHA was used as standard.

2.5 Determination of total phenolic content

Total phenolic content (TPC) was determined spectrophotometrically according to the Folin–Ciocalteu procedure [7] with minor modifications [8]. Briefly, 500 µl of extract was mixed with 2.5 mL of Folin–Ciocalteu reagent (10× dilution) and allowed to react for 5 min. Then 2.5 mL of Na_2CO_3 7.5% solution was added and allowed to stand for 15 min at 45 °C and 30 min at room temperature, before the absorbance being determined at 765 nm using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). A calibration curve for the standard (gallic acid) was used to obtain a correlation between sample absorbance and standard concentration (linearity range = 5–100 µg/mL, $R^2 > 0.998$). The total polyphenol content (TPC) of the extracts was expressed as mg of gallic acid equivalents (GAE) per gram of plant material on dry basis (db).

3. RESULTS AND DISCUSSION

3.1. DPPH free radical scavenging assay

The pre-screening study regarding the best solvent was done with Minho Shell. Table 1 summarizes the results.

Table 1 - Total phenolic content of Minho Shell regarding the use of different solvents. Results are expressed as mean ± SD of three determinations. * - means statistical differences ($p < 0.05$)

By-product extract	Solvent	TPC (mg GAE/g db extract)
Shell (Minho)	Aqueous	16.44 ± 0.83
	Hydroalcoholic	241.91 ± 0.26*
	Alcoholic	19.39 ± 1.15

According to these results, hydroalcoholic extract is the best one, being selected. The EC_{50} for DPPH scavenging activity and the results of the Folin Ciocalteu assay for the hydroalcoholic extracts are presented in Table 2. Minho showed the highest activity for shell, followed by Beira-Alta and Trás-os-Montes. However, there is no statistical different between regions ($p < 0.05$). Regarding bur, the EC_{50} varied between 38.67 mg to 76.86 µg/mL for Minho and Trás-os-Montes, respectively. Considering TPC, the content is considerable higher for shell than bur ($p < 0.05$). Regarding shell, the TPC content for Trás-os-Montes is the highest, followed by Minho and Beira-Alta. The worst TPC content was obtained with bur from Trás-os-Montes with 56.2mg GAE/g db extract. Our results are in agreement with the results previous

obtained by Vasquez *et al.* and Barreira *et al.* [9-11]. The differences observed between regions are probably due to climatic conditions and genetic variability.

Table 2 - DPPH scavenging activity (IC50) and total phenols of the tested extracts. Results are expressed as mean ± SD of three determinations.

By-product extract	TPC (mg GAE/g db extract)	DPPH EC_{50} (µg/mL)
Shell (Minho)	241.91 ± 0.26	37.61 ± 5.08
Shell (Beira-Alta)	143.02 ± 0.57	36.81 ± 4.55
Shell (Trás-os-Montes)	796.79 ± 1.07	31.80 ± 1.13
Bur (Minho)	85.28 ± 0.70	38.67 ± 6.54
Bur (Beira-Alta)	92.24 ± 1.19	76.86 ± 4.04
Bur (Trás-os-Montes)	56.20 ± 3.47	57.61 ± 9.29

4. CONCLUSION

Castanea sativa Mill by-products hydroalcoholic extracts presented a high potential to be explored as a sustainable source of bioactive compounds by different industries such as food, cosmetic or pharmaceutical.

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Sensory evaluation of ready-to-eat cabbage and green beans in MAP: correlation among mineral, bioactive and textural composition

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Keywords: Ready-to-eat; mineral composition; bioactive compounds; sensory evaluation

ABSTRACT

The sensory quality of minimally processed (MP) and pre-cooked (PC) green beans and cabbages, stored in MAP, was studied and correlated to data from some physicochemical parameters, bioactive compounds and mineral contents.

Samples were stored for a period of 17 and 28 days, respectively. Gas composition introduced inside the package was: $10\%O_2+45\%CO_2+45\%N_2$ for MP vegetables and $0\%O_2+40\%CO_2+60\%N_2$ for PC ones.

Sensory evaluation was carried out by a trained panel performing a quantitative descriptive analysis (QDA) using a 13 point scale to quantify attributes related to: general appearance, color, flavor (aroma and taste) and texture (hardness and mouth feel perception). Minerals (Ca, P, Mg, Na, K, Fe, Cl and S) and microstructure were analyzed by scanning electron microscopy. The studied bioactive compounds were total phenols, total flavonoids, carotenoids and anthocyanins. Firmness was also evaluated by a compression test. Data mining was carried out by canonical correlation analysis (CCA) to investigate how bioactive, firmness, moisture and mineral contents were correlated to the sensory characteristics over the storage period. Mineral losses were very small over storage time and correlated with sensory data. CCA outputs show that PC samples are grouped due to Na and Cl contents, added as NaCl in cooking procedures. Samples with higher moisture surface and brightness also presented losses of Mg. MP cabbages show a better retention of P, S and Ca. Although moisture content is related to mineral loss, no impacts on flavor were found.

1. INTRODUCTION

Nowadays, consumers demand for healthy, tasty and convenient products. Thus, food safety and security should be of major concern when preservation methods are applied. Modified atmosphere packaging (MAP) of ready-to-eat (RTE) vegetables has been extensively used once it is recognized as a preservation procedure. Consumption of vegetables is highly

recommended due to the high content of biologically active compounds with health-promoting effects, which are also relevant to the appearance, taste and flavor [1]. Vegetables are also excellent dietary sources of minerals but little attention has been paid to this issue [2]. This work evaluated the sensory quality of minimally processed (MP) and pre-cooked (PC) green beans and cabbage, in MAP, and the influence of some physicochemical parameters, bioactive compounds and mineral contents.

2. MATERIAL AND METHODS

2.1. Samples and sampling

Cabbages (Cb) and green beans (Gb), where subjected to two different treatments: minimal processing and pre-cooking, followed by packaging in a modified atmosphere and cold storage (5ºC) during 17 and 28 days, respectively. Gas composition introduced inside the packages was 10 $\%O_2$ + 45 $\%CO_2$ + 45 $\%N_2$ for MP samples and 0 $\%O_2$ + 40 $\%CO_2$ + 60 $\%N_2$for PC samples. Physicochemical, texture and sensory parameters were evaluated at 0, 5, 10, 14 and 17 days after packaging in the case of MP products and at 0, 5, 14, 20, 24, and 28 days after packaging in the case of PC vegetables.

2.2.Quality evaluation

The development of off-flavours and changes in the visual appearance of products was checked and recorded immediately after opening the package at each sampling time. Sensory quality control of the product over storage was carried out by a 15 element trained panel performing a quantitative descriptive analysis (QDA). Training was carried out until a final list was created with 17 attributes sorted into sensory groups: general appearance, colour, flavour (aroma and taste) and texture (hardness and mouth feel perception). 13 point scales were used to quantify each attribute.

The texture parameter firmness, defined as the maximum force exerted in compression, was determined using a TA-XT2i (Stable Micro Systems Ltd., U.K.) equipped with a 2 mm diameter cylindrical stainless steel probe. The compression test speed was $1mm.s^{-1}$. For each sample, about 20 measurements were performed.

Minerals (Ca, P, Mg, Na, K, Fe, Cl and S) and microstructure were analyzed by scanning electron microscopy using a Hitachi SU1510 (Japan) coupled to a Si-based semiconductor X-ray detector Bruker NanoXFlash 5010 (UK) for microanalysis of minerals. Before SEM analysis, samples were freeze dried and coated in a rotary pumped carbon coater (Quorum Q150R E, UK.).

The bioactive compounds evaluated were: total phenolic compounds, total flavonoids, carotenoids and anthocyanins. For these determinations samples were freeze dried and, except in the case of total carotenoid content, methanolic extracts were prepared according to Porter [3]. Total phenolic compounds of the extracts were determined by the Folin-Ciocalteau colorimetric method [4] with gallic acid as a standard. Anthocyanins were determined as

described by Li et al., with modifications. 2.5 ml of sample extract were dissolved in 45 ml of methanol, then placed in a ultra-sound bath for 10 min, and heated at 40 ºC during 30 min. Absorbencies were read at 528 nm [5]. Total flavonoids content was determined by a colorimetric method (510 nm). Total carotenoid quantification was performed by spectrophotometry UV / Vis, at a wavelength of 450 nm, using petroleum ether as white standard as described by Yuan, et al. [6].

3.RESULTS AND DISCUSSION

Results from all parameters evaluated are summarized in Table 1. Mineral composition by SEM analysis is a practical method enabling rapid identification of mineral phases. It uses integrated computer software that can produce information on mineral composition and distribution along the images generated by the scanning electron microscope. Figure 1 is an example of the spectra obtained during the observation of a PC cabbage sample with five days of storage.

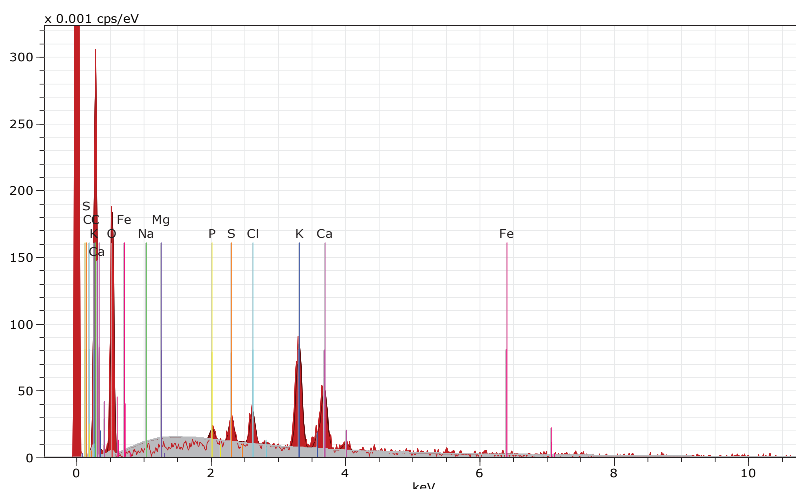

Figure 1. Spectra of mineral composition in PC cabbage samples with five days of storage.

This information is of the qualitative type, but performing an adequate number of replications with appropriated statistical analysis, it can rapidly give useful information about mineral distribution along the microstructure and about mineral losses during processing or storage as in this study. Comparing MP with PC samples loss of mineral contents were observed.

Physical properties (firmness) and the levels of bioactive compounds, studied over storage, did not present a clear tendency (decrease or increase levels). According to the results of Table 1 it can be shown that plant tissue reacts against the MAP conditions but generally no significant differences are observed over storage time. This was also felt by the panelists that performed the sensory analysis.

Tabela 1. Mineral composition, firmness, moisture content (MC), total carotenoid (TC), total phenolic compounds (TPC), anthocyanins (ANT) and flavonoid (FL) content of MP and PC cabbage and green beans over storage time.

	Code	Time (days)	Ca %	P %	Mg %	Na %	K %	Fe %	Cl %	S %	Firmness N	MC %	TC µg/g	TPC mgGAc/Lext	ANT mg/lext	FL mg/lext
MP	Cb0	0	2.53	2.33	0.05	0.60	0.16	nd	0.77	0.58	12.28	95.78	3.00	193.65	107.29	267.19
	Cb1	5	6.21	7.89	nd	nd	0.41	0.22	1.73	1.06	19.64	94.90	0.45	174.38	102.70	81.25
	Cb2	10	1.58	3.58	nd	nd	0.11	0.30	0.38	0.62	12.24	95.21	0.12	90.00	107.71	43.75
	Cb3	14	2.66	3.75	nd	0.06	0.32	0.16	0.44	0.74	11.14	96.23	0.17	94.17	115.22	75.00
	Cb4	17	4.29	3.70	nd	nd	0.28	nd	0.88	0.62	8.59	95.65	0.21	98.33	106.87	43.75
PC	Cb*0	0d	1.26	0.34	0.11	2.85	2.28	0.87	5.01	0.66	0.88	94.09	1.48	183.33	91.01	160.94
	Cb*1	7	0.77	0.17	0.14	2.42	1.75	0.66	3.66	0.51	0.25	95.16	0.89	186.46	101.86	228.13
	Cb*2	14	0.81	0.20	0.04	2.55	1.53	0.32	3.32	0.53	0.39	94.50	3.03	165.63	93.51	253.13
	Cb*3	20	0.80	0.29	0.09	2.95	1.73	0.25	3.62	0.54	0.89	94.35	3.95	177.08	103.53	253.13
	Cb*4	24	0.88	0.14	0.00	3.09	1.26	nd	4.27	0.27	0.57	95.14	1.90	132.29	91.84	212.50
	Cb*5	28	0.61	0.20	0.12	3.23	1.51	0.48	4.03	0.68	0.55	94.48	2.65	132.29	91.84	240.63
MP	Gb0	0	0.52	0.54	0.17	0.14	2.67	0.17	0.16	0.16	38.43	94.93	5.30	81.24	109.38	84.38
	Gb1	5	1.03	0.13	0.32	0.14	1.96	0.17	0.23	0.11	64.44	95.05	1.87	94.00	99.36	68.75
	Gb2	10	0.70	0.28	0.30	0.01	4.10	1.00	0.51	0.26	40.73	93.79	4.05	115.35	101.86	103.13
	Gb3	14	1.22	0.37	0.25	0.15	2.72	0.40	0.37	0.15	33.26	95.06	2.23	88.06	107.71	59.38
	Gb4	17	1.19	0.22	0.24	0.14	1.59	nd	0.09	0.16	27.34	93.87	3.38	117.23	110.21	184.38
PC	Gb*0	7	0.19	0.05	nd	1.85	0.40	0.27	3.47	0.06	8.90	93.00	31.91	66.09	92.26	134.38
	Gb*1	14	0.39	0.12	0.19	3.17	0.92	0.31	4.89	0.15	5.69	94.35	7.48	57.29	96.02	90.63
	Gb*2	20	0.30	0.35	0.12	2.83	0.76	0.09	4.08	0.15	4.34	93.87	11.00	62.40	91.84	153.13
	Gb*3	24	0.24	0.25	0.25	3.65	1.02	0.43	4.67	0.08	4.08	94.20	6.10	53.44	91.84	93.75
	Gb*4	28	0.32	0.05	0.08	3.04	0.71	nd	4.50	0.04	4.27	95.41	6.83	57.60	92.68	93.75
	Gb*5	7	0.30	0.16	0.18	3.66	0.98	0.07	4.90	0.06	5.08	93.85	11.44	58.33	95.18	81.25

Data mining was carried out by a CCA. Three data matrices were arranged: (i) one relative to the sensory evaluation of all sample products over time; (ii) one relative to the analysis of some bioactive compounds, moisture content and texture; and (iii) one relative to mineral contents. Two analyses were carried out, comparing: A) sensory data *versus* bioactive compounds and moisture contents and texture, with results shown in Figure 2; B) sensory data *versus* mineral content, with results presented in Figures 3. In each analysis, CCA produces two graphs, one for each matrix. In each graph two canonical dimensions (axes) are visible (the first horizontal and the second vertical). The interpretation of each dimension was carried out in each graph using labels at the axes' edges.

It is important to note that if two graphs show a similar scatter of sample points on a left/right orientation, this reflects an important correlation described by the horizontal axes. Similarity on a top/bottom orientation reflects a second important correlation between the data sets.

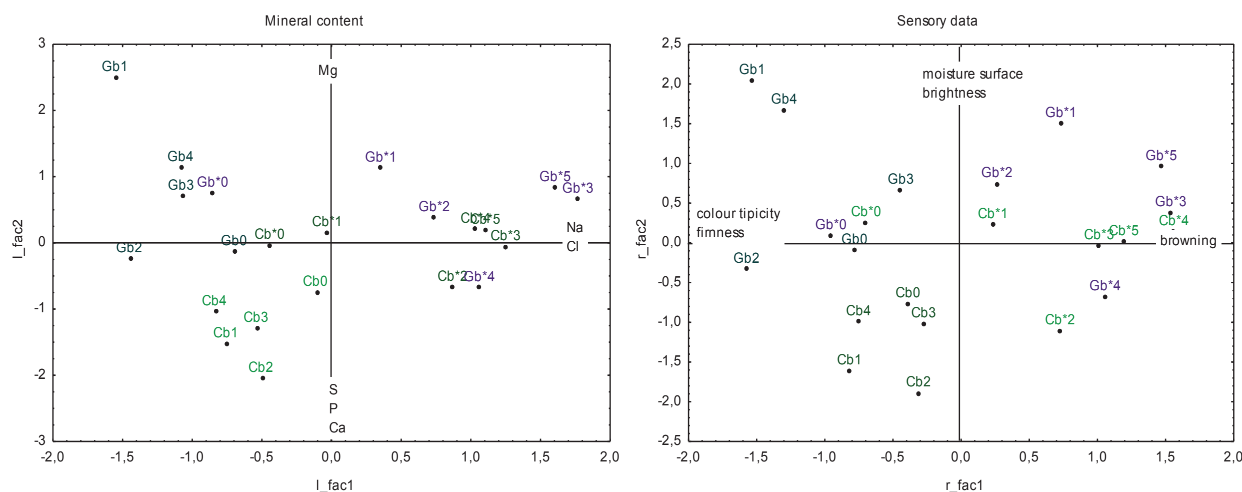

Figure 2. Canonical correlation analysis of bioactive compounds and moisture content and texture (left) related to sensory data (right).

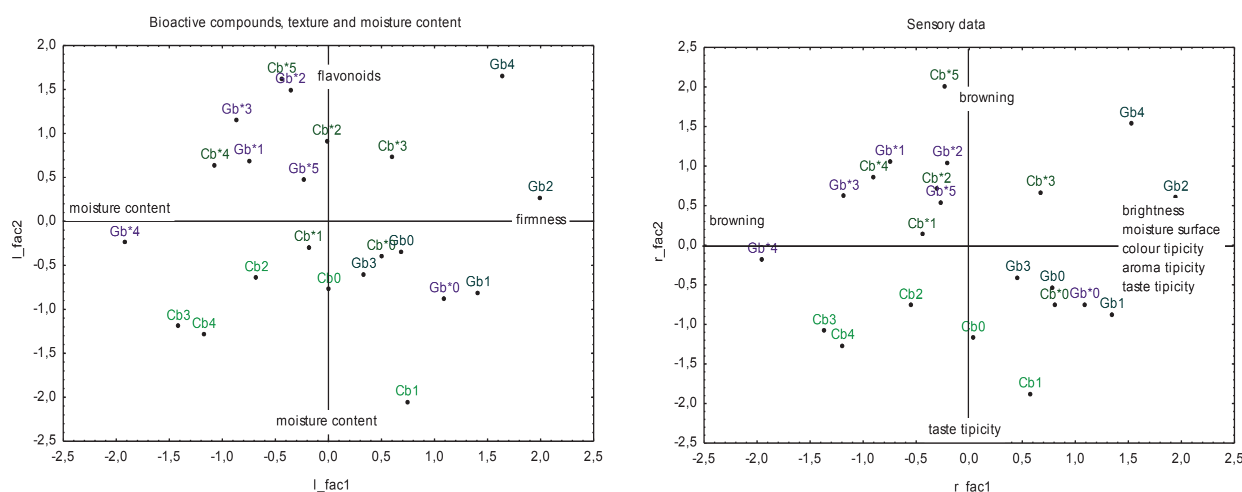

Figure 3. Canonical correlations analysis of mineral contents (left) versus sensory data (right). Legend for Figures 2 and 3: Cb and Gb (MP cabbage and green beans, respectively); Cb* and Gb* (PC cabbage and green beans, respectively); nunbers refer to sampling times.

In Figure 2, MP green beans are on the left/top and MP cabbages are on the left/bottom, the former with high Mg, surface moisture and brightness, the latter showing higher contents in P, S and Ca, being dryer and dull. This is a top/down aspect representing the second important correlation. The first correlation separates MP and PC vegetables. PC vegetables are displaced towards the right hand side of the graphs, having higher Na and Cl contents, which is correlated with browning and loss of colour tipicity and firmness.

In Figure 3 it is shown that MP cabbages are high in moisture and low in flavanoid contents, with typical taste, while MP green beans are drier, firmer, with high flavanoid content, retaining typical organoleptic characteristics over storage time. PC samples, mainly located in the top/left part of the graphs, mainly samples with higher storage time, show an increase in browning accompanied by the retention of flavanoids and an expected loss of freshness

attributes. In this case, both canonical dimensions are important to characterize fresh/cooked samples and green beans/cabbages and both are necessary to display the effect of storage time slightly affecting cooked samples.

4. CONCLUSIONS

It is possible to conclude that MAP is adequate to preserve minimally processed and pre-cooked cabbage and green bean samples. Nutritional parameters and sensory quality remained quite stable over storage, which is a good indication that producers and industry can add some days to logistic operations without compromising quality. Therefore, the possibility to extend shelf-life and add days "to best before" dates is verified. It can also be concluded that canonical correlation analysis is a useful tool to aid interpretation of great data sets, given the facility to look for correlations between different types of parameters.

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