



## Effect of cooking on total vitamin C contents and antioxidant activity of sweet chestnuts (*Castanea sativa* Mill.)

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### ABSTRACT

In this work the total vitamin C contents (ascorbic acid + dehydroascorbic acid) and antioxidant activity of raw and cooked chestnuts was evaluated. The vitamin C contents of raw chestnuts varied significantly between the different cultivars (cv) studied and it varied from 400 mg/kg dry weight (cv Lada) to 693 mg/kg dry weight (cv Martainha). The different cultivars behave differently during the cooking process concerning the loss of vitamin C. A significant decrease in the vitamin C content of the chestnuts was observed, 25–54% for the boiling process and 2–77% for the roasting process. Boiled and roasted chestnuts can be good sources of vitamin C since it may represent 22.4%, 16.2%, 26.8% and 19.4%, respectively, of the recommended dietary intake for an adult man and woman. The cooking process significantly changed the antioxidant activity of the chestnuts. A difference was observed between the cultivars during the cooking processes, concerning the antioxidant activity. For the raw chestnuts the variation in vitamin C content of the chestnuts explains 99% of the antioxidant activity variation but for the roasted and boiled chestnuts this percentage significantly decreases to 51% and 88%, respectively. Although a high antioxidant activity is still present in the cooked chestnuts, the cause for this antioxidant activity is less dependent on the vitamin C content of the chestnuts, probably due to the conversion of ascorbic acid to dehydroascorbic acid. The increase in gallic acid during the cooking process, presumably transferred from the peels to the fruit, also contributes to the high antioxidant activity observed for the cooked chestnuts.

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### 1. Introduction

Chestnut kernels are a highly appreciated nut (*Castanea sativa* Mill.) in Southern European countries, where it is a frequent ingredient of the Mediterranean diet since ancient times. In recent decades its use and consumption has increased partly due to the diversification of chestnut products available in the market, such as frozen and boiled frozen chestnuts among others. Nevertheless consumers still prefer the fresh products that they consider as having higher quality and greater positive health effects. When available in the fresh form consumption is preceded by cooking, with roasting or boiling being the most common cooking methods.

Chestnuts contains significant concentrations of health promoting compounds, including the antioxidants L-ascorbic acid (Desmaison & Adrian, 1986; Neri, Dimitri, & Sacchetti, 2010; Ribeiro et al., 2007; Vasconcelos et al., 2010), vitamin E (Barreira et al., 2009; Vasconcelos et al., 2010), carotenoids (Vasconcelos et al., 2010) and polyphenols (mainly gallic and ellagic acids) (Gonçalves et al., 2010; Vasconcelos, Bennett, Rosa, & Ferreira-Cardoso, 2007, 2009; Vasconcelos et al., 2010). Epidemiological studies indicate that fruits and vegetables have protective effects against degenerative diseases such as cancer and cardiovascular diseases (Arts & Hollman, 2005; Neuhaus, 2004). Since oxidative stress is common in chronic degenerative diseases, it has been assumed that dietary antioxidants such as those present in chestnuts, may explain this protective effect (Aruoma, 1998; Naczk & Shahidi, 2006), by preventing free radicals from damaging biomolecules such as proteins, DNA, and lipids (Halliwell, 1996). Of the tree nuts, chestnuts, walnuts and pecans have the highest content of antioxidants (Blomhoff, Carlsen, Andersen, & Jacobs, 2006). Of the various antioxidants those quantitatively more abundant in chestnuts is L-ascorbic acid, the biologically active form of vitamin C

**Abbreviations:** ABTS, 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid; d.w., dry weight; LTS, least trimmed squares; MPA, meta-phosphoric acid; PCA, principal component analysis; PDO, protected designation of origin; RDI, recommended dietary intake; TCEP, tris(2-carboxyethyl)-phosphine; TEAC, Trolox equivalent antioxidant capacity.

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(Lykkesfeldt, 2002). Vitamin C is the generic term for all compounds exhibiting the biological activity of L-ascorbic acid. L-Ascorbic and L-dehydroascorbic acid are the primary dietary sources of vitamin C (Ball, 2006). Both ascorbic acid and dehydroascorbic acid are absorbed through the gastrointestinal tract (Rumsey & Levine, 1998) and can be interchanged enzymatically *in vivo* (Wells, Xu, & Washburn, 1995). It acts as a potent, and probably the most important, hydrophilic antioxidant, and is believed to be of major importance for protection against diseases and degenerative processes caused by oxidative stress (Lykkesfeldt, 2002; Retsky, Freeman, & Frei, 1993) being effective in scavenging superoxide radical anion, hydrogen peroxide, the hydroxyl radical, singlet oxygen, and reactive nitrogen oxide (Halliwell & Gutteridge, 2007). The current recommended dietary intake for adults (RDI, representing the recommended dietary allowance) for vitamin C is determined as 90 mg/day for men and 75 mg/day for women (Institute of Medicine, 2000), although strong arguments, like the preventive functions of L-ascorbic acid in cardiovascular disease and certain cancers (Diplock et al., 1998) favours the increase in dietary intakes (Carr & Frei, 1999).

L-Ascorbic and L-dehydroascorbic acids are prone to thermal degradation (Ball, 2006). Also other factors like oxygen availability, pH, presence of other antioxidants, transition metal catalysis and the presence of ascorbic acid oxidase (Ball, 2006; Lee & Kader, 2000) influence the rate L-ascorbic acid degradation. For these reasons it is expected that the amount of vitamin C of chestnuts to decrease during the cooking procedure, and some authors argue that it would be completely lost upon cooking (Neri et al., 2010). No data is available for the vitamin C content of roasted and boiled chestnuts (ascorbic + dehydroascorbic acid). Only one paper has reported the L-ascorbic acid content of roasted, boiled and fried chestnuts, where it was found to decrease with these thermal treatments (Ribeiro et al., 2007). Nevertheless, the amount of ascorbic acid for the raw chestnuts were low 45–164 mg/kg d.w. when compared to literature values that range from 260 to 1260 mg/kg d.w. (Desmaison et al., 1986; Neri et al., 2010), probably due to the fact that the extraction conditions were not adequate to avoid L-ascorbic acid oxidation (Washko, Welch, Dhariwal, Wang, & Levine, 1992). Also the data on the antioxidant activity of chestnuts is scarce (Barreira, Ferreira, Oliveira, & Pereira, 2008) and limited to raw chestnuts. No data is available for the antioxidant activity of cooked chestnuts, the commonly consumed form of fresh chestnuts, in which this activity would also be affected by the cooking procedures.

The aims of this work were to analyse the total vitamin C contents of nine Portuguese chestnut cultivars, evaluate the effect of the most common cooking methods on the vitamin C contents and the suitability of the cooked chestnuts as a source of vitamin C. The secondary aim was to evaluate the hydrophilic antioxidant activity of the nuts and the effect of the cooking process on the antioxidant activity of chestnuts, and ascertain the contribution of vitamin C and gallic and ellagic acid to the total antioxidant activity of chestnuts.

## 2. Material and Methods

### 2.1. Reagents

Working standards of ascorbic acid (Merck) and isoascorbic acid (BDH) were used. Meta-phosphoric acid (MPA), tris(2-carboxyethyl)-phosphine hydrochloride (TCEP-HCl; >98%), trifluoroacetic acid, acetic acid, methanol p. a., 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid (ABTS) and Trolox were from Sigma–Aldrich, formic acid (pro-analysis) and acetonitrile (HPLC gradient grade) were purchased from Panreac. HPLC-grade water was prepared

with a SGS water purification system. The regenerated cellulose membrane filters were from Marchery-Nigel.

### 2.2. Sample preparation

The selection of the most important Portuguese cultivars was made within the three Protected Designation of Origin (PDO) areas for chestnut tree production in the Trás-os-Montes e Alto Douro region: PDO Terra Fria (cvs. Aveleira, Boaventura, Côtá, Lamela and Trigueira); PDO Padrela (cvs. Judia, Lada and Longal1) and PDO Soutos da Lapa (cvs. Longal2 and Martaínha). In 2006, three representative trees from each cultivar were selected and three replications of mature chestnuts (1 kg of fruits per sample) of each selected tree were randomly collected and kept at 2 °C until analysis for a maximum of 3 days.

Raw chestnuts (500 g) from each cultivar, previously cross-cut on the top, were boiled with 2 l of water and 5 g of salt for 20 min. Also, 500 g of raw nuts from each cultivar, were slit with a knife and spread with 7 g of salt, and were roasted in a preheated electric oven with air circulation (Nabertherm Mod. – L9R; Nabertherm GmbH, Lilienthal, Germany), at 200 °C for 40 min. These conditions were selected from previous tests. After processing, the samples were peeled and the nuts rapidly cut into small pieces and milled in a Model SK 100 Cross Beater (Retsch GmbH, Haan, Germany) to a uniformly fine powder. Samples were stored in a freezer at –80 °C until analyses were performed. Both boiling and roasting processes were done in triplicate.

### 2.3. Vitamin C content

Vitamin C content of chestnuts (ascorbic + dehydroascorbic acid) were performed by a validated hydrophilic interaction chromatography method described previously (Barros, Silva, Gonçalves, & Nunes, 2010). Briefly, for the analysis of freeze dried chestnuts by internal standard calibration, after weighting 2.5 g of chestnuts, 10 ml of IAA internal standard (2500 mg/l in the extraction-reduction solution) was added, followed by addition of 40 ml of the extraction-reduction solution (5% MPA, 2 mM tris(2-carboxyethyl)-phosphine (TCEP) and 2 mM EDTA), and after standing for 60 min at 40 ± 2 °C the suspension was centrifuged for 5 min at 3000g a sample was taken, filtered and analysed. A Dionex liquid chromatographic system (USA) consisting of an Ultimate 3000 pump, PDA-100 photodiode array detector, a WPS-3000TSL Analyt auto sampler and column oven (Model TCC-3200). The chromatographic analyses were carried out on an TSK-gel Amide-80 (4.6 i.d. × 100 mm, 5 µm, Tosoh Bioscience, Japan) kept at 20 °C, unless otherwise stated. The optimal analysis conditions were carried out using acetonitrile (B): aqueous 0.1% trifluoroacetic acid (A) (90:10, v/v) as a mobile phase during 7 min, changed to 80%A at 8 min and maintained until 13 min, 10%A at 14 min and maintained at 10%A until 20 min. The flow rate was 0.7 ml/min and the absorbance at 210–350 nm was monitored, and the absorbance at 244 was used for analysis.

### 2.4. Hydrophilic antioxidant activity

For the determination of the antioxidant activity a 60% methanol aqueous solution was used as the extraction solvent. Preliminary experiments showed that four extractions (shaking during 30 min) with 10 ml of the extraction solution each were needed to extract all the measurable antioxidant activity from 0.5 g of chestnuts. After each extraction the dispersion was centrifuged at 6000 rpm during 10 min, and the solution was decanted. The extraction solutions were combined and the volume was adjusted to 50 ml with the extraction solution. The radical-scavenging activity was determined soon after extraction by the 2,2-azino-

bis(3-ethylbenzothiazoline)-6 sulphonic acid (ABTS) radical cation decolourisation assay, as described by Ozgen, Reese, Scheerens, Tulio Jr. and Miller (2006). For the assay, ABTS<sup>•+</sup> radical was prepared by mixing an ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture was allowed to stand for 12–16 h at room temperature in the dark until reaching a stable oxidative state. The ABTS<sup>•+</sup> solution was diluted with 20 mM sodium acetate buffer (pH 4.5) to an absorbance of  $0.70 \pm 0.01$  at 734 nm. The reaction was started by the addition of 50, 100 and 200  $\mu$ l of the 60% methanolic extract of the chestnuts diluted conveniently to 2.00 ml of the diluted ABTS<sup>•+</sup> solution. ABTS<sup>•+</sup> bleaching was monitored at 734 nm at 25 °C for at least 30 min and the percentage of discoloration after 15 min was used as the measure of antioxidant activity. The ABTS<sup>•+</sup> bleaching was proportional to the concentration of the sample added to the medium. The antioxidant activity of the extract was calculated as Trolox equivalent antioxidant capacity (TEAC) and was expressed as mmoles of Trolox equivalents per kg of sample. A standard curve of the percentage of ABTS<sup>•+</sup> inhibition in function of Trolox concentration (0.11 mM to 0.014 mM in 60% aqueous methanol) was used for the calculations.

For determination of the relative ABTS<sup>•+</sup> discoloration ability of pure ascorbic acid, gallic acid, ellagic acid and Trolox, properly diluted standard solutions were tested at two different pH's: pH 4.5, by the method described previously, and pH 7.4 by the method described by Rice-Evans, Miller, and Paganga (1996) being different from the method described previously by the fact that the ABTS radical solution was prepared with PBS (pH 7.4) to an absorbance of  $0.70 \pm 0.01$  at 734 nm.

### 2.5. Statistical analysis

All analysis (extraction and purification) were performed in triplicate; each replicate was quantified in duplicate. Data were expressed as means  $\pm$  standard deviation. Two-way ANOVA analysis of the experimental data was conducted to estimate the effects of the cultivar, the cooking treatment and their interaction. Tukey HSD post-hoc test was used to indicate significant differences between means at the 5% level. Principal-component analysis (PCA) on correlation matrix with Varimax rotation was applied to describe the data set and to detect the most important variables for determining the data structure. All statistical treatments were performed on the Statistica 7 (StatSoft, Inc., Tulsa, Oklahoma, USA), statistical software.

Robust regression was performed by least trimmed squares (LTS) (Rousseeuw & Annick, 1987) in order to obtain resistant linear slopes and to detect deviant observations on the data set. The LTS regression minimises the sum of the trimmed squared residuals. Standardised robust residuals were considered deviant if they were  $e_i \geq 2.5$ . Calculations were performed with ltsreg algorithm of the robustbase library of the R version 2.10.1 (Copyright (C) 2009 The R Foundation for Statistical Computing).

## 3. Results and discussion

### 3.1. Vitamin C content of raw and cooked chestnuts

The vitamin C content of chestnuts was determined as the sum of ascorbic and dehydroascorbic acid, after chemically reducing the dehydroascorbic acid to ascorbic acid with tris(2-carboxyethyl)-phosphine (Fig. 1) (Barros et al., 2010). The vitamin C content of raw chestnuts varied significantly between the different cultivars studied (Table 1), and it varied from 400 mg/kg dry weight (d.w.) in cv Lada to 693 mg/kg d.w. in cv Martaínha. These values are in the range of the ascorbic acid concentrations reported in the liter-

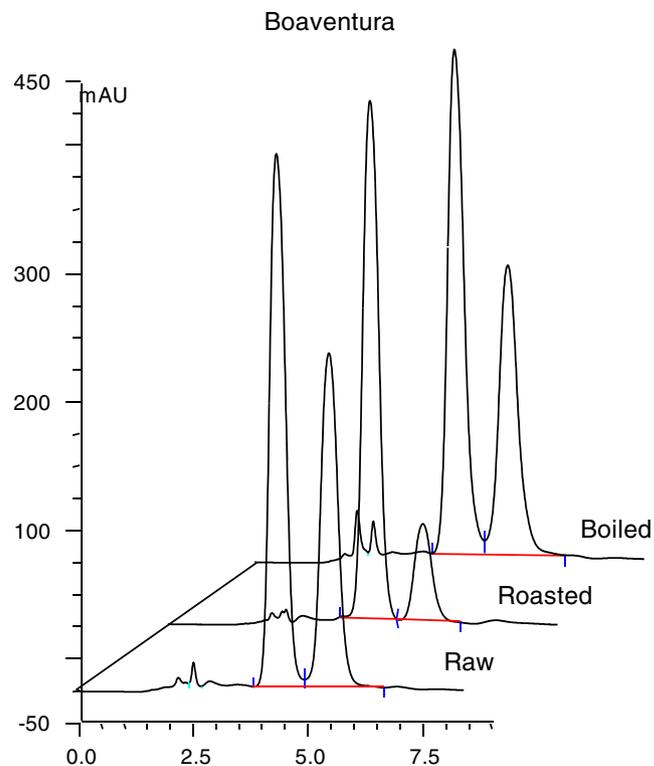


Fig. 1. Chromatographic determination of total ascorbic acid after reduction by TCEP (vitamin C) by the internal standard method by hydrophilic interaction chromatography on TSK-gel Amide 80.

ature (Desmaison et al., 1986; Neri et al., 2010). On a fresh basis the content is approximately half of the dry weight and varies between 160 and 296 mg/kg, a value lower than that found for the citrus fruits (300–500 mg/kg) and in the range of values found on potatoes (100–300 mg/kg) (Ball, 2006). Martaínha, Lamela, Trigueira, Cota and Longal2 presented vitamin C contents not significantly different from each other, with values ranging from 550 to 693 mg/kg d.w., and Boaventura, Longal, Lada and Judia contained significantly lower values that ranged from 400 to 488 mg/kg d.w. It is known that cultivars can have a dramatic effect in the vitamin C content of the horticultural crops, nevertheless other factors including sunlight, maturity stage, post-harvest processing and storage can also significantly affect the vitamin C content of fruits and vegetables (Ball, 2006; Lee & Kader, 2000). As the cultivars studied originated from different locations, and were harvested in different periods according to their commercial maturity, no definitive conclusion can be drawn about the cultivars with higher vitamin C content.

The cooking processes resulted in a significant decrease in the vitamin C content of the chestnuts (ANOVA  $p < 0.00001$ ). In average a decrease of 37% (25–54%) for the boiling process and 33% (2–77%) for the roasting process was observed. There was a significant interaction between the cultivars and the cooking process, meaning that the cooking process resulted in a different variation in the vitamin C content of the different chestnuts cultivars. Two different behaviours were observed for the cultivars studied. In the first group of cultivars consisting of Martaínha, Lamela, Trigueira, Boaventura and Longal2, the boiling and roasting process resulted in a significant decrease in the vitamin C content of the chestnuts (41% decrease for the boiling process and 53% for the roasting process). For the Martaínha, Lamela and Longal2, no significant differences in the vitamin C losses were observed between the boiling and roasting process (46% for boiling and 41% for roasting), but for the Trigueira and Boaventura cultivars the

**Table 1**  
Vitamin C, Gallic and Ellagic acid (mg/kg) and Antioxidant Activity of Raw (mmol Trolox/kg), boiled and roasted chestnuts.

Cultivars	Cooking	Water (%)	Vitamin C		TEAC		Gallic acid <sup>a</sup>	Ellagic acid <sup>a</sup>
Lamela	Raw	54.0	644 ± 61 <sup>b</sup>	A <sup>c</sup>	0.997 ± 0.034	A	12.5	6.4
	Roasted	45.1	235 ± 16	A	0.705 ± 0.014	A	8.4	5.1
	Boiled	57.2	298 ± 26	A	0.814 ± 0.050	A	12.1	9.9
Trigueira	Raw	52.5	560 ± 38	A	0.864 ± 0.008	A	4.4	2.9
	Roasted	44.9	191 ± 21	A B	0.711 ± 0.007	A B	6.2	9.1
	Boiled	56.6	359 ± 23	A B	0.894 ± 0.073	B	15.5	9.3
Cota	Raw	50.7	579 ± 38	A	0.910 ± 0.019	A	5.4	1.6
	Roasted	44.1	491 ± 48		0.943 ± 0.017		14.2	6.9
	Boiled	55.3	434 ± 35	A	1.041 ± 0.022	A	13.5	9.8
Boaventura	Raw	50.6	425 ± 21	A	0.670 ± 0.009		10.2	13.4
	Roasted	46.2	97 ± 9	A B	0.570 ± 0.017	A	5.8	6.3
	Boiled	55.6	301 ± 21	A B	0.703 ± 0.014	A	8.9	7.2
Longal1	Raw	56.0	402 ± 18	A	0.564 ± 0.024		5.0	2.8
	Roasted	44.3	392 ± 6		0.633 ± 0.017	A	9.8	4.3
	Boiled	59.6	282 ± 10	A	0.474 ± 0.015	A	15.5	4.3
Lada	Raw	60.0	400 ± 31	A	0.647 ± 0.005	A	3.8	n.d.
	Roasted	51.0	391 ± 31	B	0.869 ± 0.031	A B	13.1	6.9
	Boiled	62.0	269 ± 30	A B	0.686 ± 0.017	B	14.9	13.4
Judia	Raw	54.2	488 ± 65	A	0.736 ± 0.024		12.8	3.0
	Roasted	47.5	449 ± 26	B	0.735 ± 0.026		11.7	7.6
	Boiled	60.3	297 ± 17	A B	0.689 ± 0.020		12.7	12.7
Martaínha	Raw	50.0	693 ± 29	A	1.046 ± 0.103	A	13.5	8.3
	Roasted	43.3	459 ± 65	A	0.909 ± 0.033	A B	14.7	13.5
	Boiled	53.2	407 ± 20	A	0.716 ± 0.022	A B	11.0	22.0
Longal2	Raw	51.2	571 ± 40	A	0.881 ± 0.050	A	12.7	2.7
	Roasted	44.2	421 ± 55	A	0.753 ± 0.040	A	22.5	21.1
	Boiled	60.7	326 ± 42	A	0.796 ± 0.032		12.4	11.1

<sup>a</sup> Values from Gonçalves et al. (2010).

<sup>b</sup> Values represent the mean ± standard deviation ( $n = 3$ ).

<sup>c</sup> A and B are significantly different ( $p < 0.05$ ) from all other A and B, respectively, for the same cultivar.

roasting process lead to a significantly higher decrease in the vitamin C when compared to the boiling process (66% and 77% for the roasting process and 36% and 29% for the boiling process, respectively). For the second group of cultivars composed by the Cota, Longal, Lada and Judia, in contrast with the first group of cultivars, only the boiling process led to a significant decrease in the vitamin C content of the chestnuts (32% decrease for the boiling process versus 7% decrease for the roasting process). The non-significant difference in the vitamin C content of the roasted chestnuts for this group of chestnut cultivars, did not indicate that no transformation of ascorbic acid to dehydroascorbic acid had occurred, since the method used by the group, for the analysis of vitamin C uses reducing conditions, allowing recovery of the two vitamins. The reason for this different behaviour between the two groups of cultivars during the different cooking processes is not clear, but a variety of reasons can contribute to this different behaviour namely: different thermal transfer properties of the shell and peel, different thermal diffusivity coefficient of the chestnuts. Also a different mineral content of the different chestnuts can be a factor influencing this different behaviour of the chestnuts during the cooking process, as it is known that metals can catalyse the degradation of ascorbic acid (Ball, 2006). A different behaviour in the thermal processing of different chestnuts cultivars concerning flavour characteristics has been observed (Kunsch et al., 2001).

From these results it can be concluded that boiled and roasted chestnuts can be good sources of vitamin C. For boiled chestnuts in average the vitamin C content of 100 g of chestnuts corresponds to 22.4% of the RDI for adult man and 26.8% for adult woman and for the roasted chestnuts in average 16.2% of RDI for adult man and 19.4% of the RDI for adult woman.

### 3.2. Hydrophilic antioxidant activity

The hydrophilic antioxidant activity of the raw, boiled and roasted chestnuts is presented in Table 1. For the raw chestnuts, the hydrophilic antioxidant activity is dependent on the cultivar

and varies from 0.546 for Longal1 to 1.042 mmol Trolox/kg dry weight for Martaínha. The cultivar Boaventura, Longal1, Lada and Judia contained the lowest antioxidant activity that ranged from 0.546 to 0.736 mmol Trolox/kg d.w., and the cultivars from Martaínha, Lamela Trigueira, Cota and Longal2 presented the highest that varied from 0.864 to 1.042 mmol Trolox/kg d.w. The antioxidant activity of the raw chestnuts are lower than that obtained in a previous study for raw Italian chestnut cultivars (Neri et al., 2010) using the same antioxidant assay. They found TEAC values from 3.02 to 3.11  $\mu$ moles Trolox equivalents  $g^{-1}$  fresh weight. This difference can be attributed to a different content in antioxidant metabolites for the Italian cultivars studied or to the different pH of the buffer used in the ABTS assay (pH 7.4 vs pH 4.5 in our work, see below). The cooking process changed significantly the antioxidant activity of the chestnuts. A factorial ANOVA showed that a significant interaction occurred between the cultivars and the cooking process ( $p < 0.0001$ ) in relation to the antioxidant activity. This indicates a different behaviour between the cultivars during the cooking processes concerning the hydrophilic antioxidant activity. The behaviour of the different cultivars concerning the change in antioxidant activity with the cooking treatment is substantially more complex than that observed for the vitamin C content described previously. For the cultivars Boaventura, Longal1 and Judia, the cooking process, either boiling or roasting, did not change significantly the antioxidant activity of the chestnuts when compared to the raw. For the Judia cultivar, no significant difference was observed for the roasted and boiled chestnuts. Nevertheless, the antioxidant activity of the boiled Boaventura chestnuts was significantly higher than roasted chestnuts, the reverse being observed for the Longal1 cultivar.

For the Cota cultivar only the boiling process changed significantly the antioxidant activity of the chestnuts, where an increase in the antioxidant activity was observed. There was no significant difference between the raw and roasted Cota cultivars.

For the Trigueira, Lada and Longal2 cultivars, only the roasting process resulted in a significantly different antioxidant level of the chestnuts when compared to the raw chestnuts. However for

the Trigueira and Longal2 cultivars the antioxidant activity of roasted chestnuts decreased when compared to the raw chestnuts (–16% in average), but for the Lada cultivar the roasting process resulted in a higher antioxidant activity (+34%). For the Martainha and Lamela cultivars, both cooking treatments lead to a significantly different antioxidant activity where both cooking treatments reduced the antioxidant activity of the chestnuts (–23% in average). The TEAC values obtained for the boiled and roasted chestnuts represents a median value between the TEAC values of other fruits and vegetables (Pellegriani et al., 2003), showing that cooked chestnuts can make a significant contribution to the antioxidant intake.

### 3.3. Contribution of vitamin C and polyphenolic compounds to the hydrophilic antioxidant activity

As can be observed in Fig. 2, there was observed a significant correlation between the vitamin C content of the raw and cooked chestnuts and the antioxidant activity. A robust regression was performed based on the least trimmed squares (LTS) that allowed us to identify three deviant observations, one in the raw chestnuts group, Longal1, and two on the boiled chestnuts group, Longal1 and Martainha. For these cultivars the observed antioxidant activity was lower than what would be expected from their vitamin C contents as observed for the other cultivars. These deviant observations can be due to a series of reasons, namely a higher amount of dehydroascorbic acid in these samples, or being due to the fact that the measure hydrophilic antioxidant activity is in fact the sum of all the antioxidant and pro-oxidant activity of the chestnut extract obtained. Some pro-oxidants that can be present are the metal ions like Fe and Cu that are present in the chestnuts (Vasconcelos, Bennett, Rosa & Ferreira-Cardoso, 2010). Their pro-oxidant activity is in turn affected by the presence of carboxylic acids like citric and malic, acids known to be present in the raw, but increase for the roasted chestnuts (Gonçalves et al., 2010). Also ascorbic acid is not the only antioxidant in the chestnuts as polyphenolic compounds, mainly gallic and ellagic acids are also present (Gonçalves et al., 2010; Vasconcelos, Bennett, Rosa & Ferreira-Cardoso, 2010). For the raw chestnuts the variation in vitamin C content of the chestnuts explains 98.97% of the variation of antioxidant activity of the chestnuts. This value decreases to 87.93% for the boiled chestnuts and 50.74% for the roasted chestnuts. Also the slopes of the regression lines obtained for the boiled

and roasted chestnuts differed from that obtained for the raw chestnuts (Fig. 2), being lower for the roasted and higher for the boiled chestnuts. This means that for the roasted chestnuts, for the same vitamin C content a lower antioxidant activity is present in the extract. Although for the roasted chestnuts the decrease in the slope is understandable as a thermal conversion of the ascorbic acid to dehydroascorbic acid and also irreversible conversion of dehydroascorbic acid to 2,3-diketoglulonic acid, and as for the vitamin C content, both ascorbic acid and dehydroascorbic acid are quantified, for the *in vitro* antioxidant activity, only the ascorbic acid makes a contribution to the measured TEAC. The higher slope of the boiled chestnuts probably means that additionally to the ascorbic acid, other compounds are contributing significantly to the measure TEAC. The contribution of other compounds to the TEAC is reinforced by the fact that for the boiling process, as referred to previously, the vitamin C content decreases an average of 37% for all cultivars, but only an 8% decrease of the TEAC is observed. For the roasted chestnuts the same trend is observed, an average 30% decrease in the vitamin C content, but only a 4% decrease in the antioxidant activity. Indeed for the Cota and Lada cultivars the boiling and roasting process, respectively, led to a significantly increase in the antioxidant activity. These results show that for the cooked chestnuts, although a decrease in vitamin C content is observed, a high antioxidant activity is still present in the chestnuts, and so for the cooked chestnuts, the vitamin C content can not explain alone the observed antioxidant activity. The amount of total phenolic compounds and gallic and ellagic acids for these cultivars were reported previously (Gonçalves et al., 2010), and are presented in Table 1. With a few exceptions, the amount of these two phenolic acids increases with the cooking treatment. The amount of these two phenolic acids is in average  $14.5 \pm 0.76$  mg/kg d.w. for the raw chestnuts and increases to  $21.3 \pm 0.94$  mg/kg d.w. for the roasted chestnuts and to  $25.1 \pm 0.53$  mg/kg d.w. for the boiled chestnuts (Gonçalves et al., 2010). Also the total phenolic compounds determined by the Folin–Ciocalteu method, varied from 1.1 to 0.7 g/kg (Gonçalves et al., 2010). Also the malic and citric acid content of the chestnuts can modulate the pro-oxidant activity of metal ions, acids known to be present in the raw, but increase for the roasted chestnuts (Gonçalves et al., 2010). In order to gain some insight into the complex relations between the *in vitro* antioxidant activity and the antioxidant components of the chestnuts, especially for the boiled and roasted chestnuts, a principal component analysis was performed, in order to extract the most relevant variations in the data set and variable correlations. As can be observed in Fig. 3A, the gallic and ellagic acid contents of chestnuts are highly correlated with factor 1 and the TEAC and vitamin C are highly correlated with factor 2. Total phenolics and malic acid are highly correlated with factor 3 and citric acid is correlated with factor 4 (not shown). These four factors account for 84% of the variability of the original data set. The analysis of the factor scores of the chestnut samples in the space defined by factor 1 and factor 2 (Fig. 3B), shows that the chestnuts are aligned on factor 2 according to their TEAC value and on factor 1 according to their content on gallic and ellagic acids. It can also be observed that for most of the chestnuts, although their TEAC lowers, their content in gallic and ellagic acid increases (shown by the arrows that start on the raw chestnuts for some cultivars and cooking processes), and this may impair the decrease in the TEAC that should be observed if vitamin C was the only contributor to the antioxidant activity.

In order to ascertain the contribution of the polyphenolic compounds quantified in the raw and cooked chestnuts, to the observed antioxidant activity, the relative ABTS<sup>•+</sup> discolouration ability of Trolox, ascorbic acid, gallic and ellagic acids were determined (Table 2). At pH 4.5, gallic acid had the highest ABTS<sup>•+</sup> discolouration ability followed by Trolox and ascorbic acid, the last

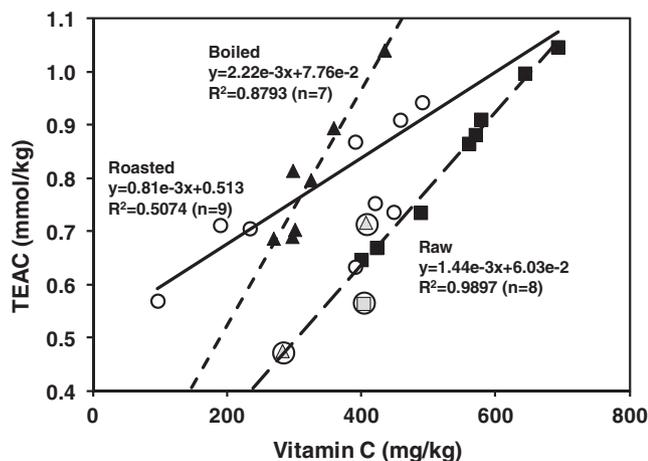
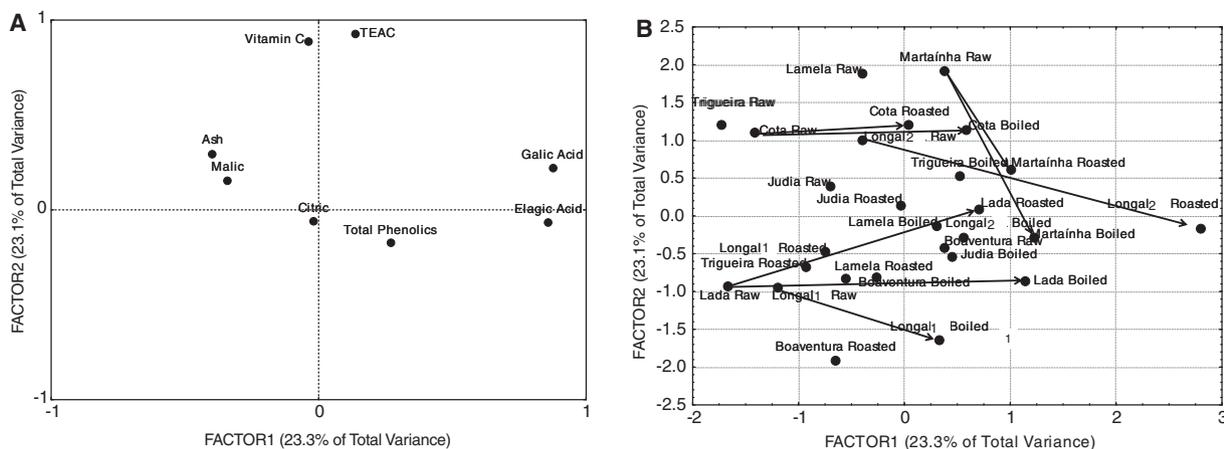


Fig. 2. Correlation between the hydrophilic antioxidant activity and Vitamin C of raw, roasted and boiled chestnuts and outlier detection by LTS regression. A good linearity of the two values illustrates that vitamin C are responsible for the TEAC of raw and cooked chestnuts.



**Fig. 3.** Results of principal components analysis. (A) Loading plot projected on the space of PC1 vs. PC2. (B) Scores plot of the chestnuts samples projected on the space of PC1 vs. PC2. Values of gallic, ellagic, citric and mallic acids, ash and total phenolics from Gonçalves et al. (2010).

two compounds showing approximately the same ABTS<sup>+</sup> discolouration ability, and surprisingly at last ellagic acid. This last result is not in accordance with the literature, where ellagic acid presents a relative antioxidant activity higher than gallic acid, Trolox and ascorbic acid (Kim & Lee, 2004; Rice-Evans et al., 1996). Nevertheless the literature values are all reported for a pH 7.4. So the ABTS<sup>+</sup> discolouration ability of the same compounds were measured at pH 7.4. As can be seen in Table 2 the values obtained for gallic acid, Trolox and ascorbic acid were similar to that obtained at pH 4.5, but the value obtained for ellagic acid was significantly higher at pH 7.4 in relation to pH 4.5, and at pH 7.4 became the first most antioxidant compound, with a relative antioxidant activity compared to gallic acid of 1.65. The different ABTS discolouration ability of ellagic acid at the two pH's may be due to the fact that ellagic acid exists as a full protonated molecule at the pH < 0.5, as a mixture of full protonated and singly deprotonated molecule in the pH region between 0.5 and 5.6, and as doubly deprotonated molecule at pH > 5.6 (Hasegawa et al., 2003). From this data the percentage of the chestnuts antioxidant activity contributed by gallic and ellagic acid were calculated (Fig. 4), assuming a 100% extraction from the chestnuts by the method applied. The contribution of ascorbic acid was not calculated as the values of vitamin C obtained in this work correspond to the sum of ascorbic and dehydroascorbic acid, and this last compounds does not presents any ABTS<sup>+</sup> discolouration ability. As can be seen in Fig. 4, for the raw chestnuts, gallic acid accounts in average for 18 ± 7% of the observed antioxidant activity and ellagic acid only for 1.4 ± 0.3%. The contribution of gallic acid to the antioxidant activity increases in average to 25% and 29% for the roasting and boiling process, respectively. The contribution of ellagic acid remains nearly constant for the roasted and boiled chestnuts, contributing only to 1.5% of the observed TEAC. Nevertheless, a high percentage of the antioxidant activity observed for the raw chestnuts (80 ± 7%) is not accounted for in these

polyphenolic compounds, certainly being due to the ascorbic acid. This value decreases to 73% and 70% for the roasted and boiled chestnuts, respectively.

The results obtained for the contribution of the gallic acid to the observed TEAC of the cooked chestnuts reinforces the importance of this polyphenolic compound to the TEAC of the cooked chestnuts. For example, for the Lada cultivar the roasting process lead to a significant increase in the TEAC of the chestnuts. The decrease in vitamin C content is negligible, but there is observed an increase in the amount of gallic acid in the chestnut from 3.8 to 13.1 mg/kg d.w. For this cultivar the gallic acid only accounts for 12% of the observed TEAC for the raw chestnut, and although the antioxidant activity increases with the roasting process, gallic acid accounts for 24% of the TEAC observed. Also for this cultivar the boiling process leads to a significant decrease in the vitamin C content of the cultivar, nevertheless the TEAC is not significantly different from the raw chestnuts, and gallic acid corresponds to 35% of the observed antioxidant activity, as its amount increases significantly for the boiled chestnut (15.0 mg/kg d.w.). The same behaviour is observed for the Cota cultivar. The TEAC for the roasted cultivar is not significantly different from the raw chestnuts, although a decrease in the vitamin C content is observed, nevertheless the amount of gallic acid increases significantly for the boiling process (5.4 and 14.4 gallic acid mg/kg d.w. for the raw and roasted chestnuts respectively), and corresponds to 24% of the observed antioxidant activity. For this cultivar, the boiling process leads to an increase in the antioxidant activity, although the amount of vitamin C decreases for the boiling process, nevertheless there is an increase in the gallic acid content of the boiled chestnut (13.5 mg/kg d.w.). On the other hand for the Martainha cultivar, both cooking treatments lead to a significant decrease in the observed antioxidant activity, and this is closely followed by a decrease in vitamin C content. For this cultivar the amount of gallic acid does not

**Table 2**  
ABTS discolouration ability of the main antioxidants present in chestnuts (% ABTS inhibition/mmoleL<sup>-1</sup>).

	0.1 M sodium acetate buffer pH 4.5			0.1 M sodium phosphate buffer pH 7.4			1	2.43	1007 <sup>b</sup>
	<i>m</i>	<i>b</i>	<i>R</i>	<i>m</i>	<i>b</i>	<i>R</i>			
Trolox	300.6 ± 6.5	1.59 ± 0.48	0.9992	298.6 ± 7.4	-0.98 ± 0.55	0.9990	1	1007 <sup>b</sup>	
Gallic acid	743.5 ± 22.1	7.11 ± 1.16	0.9990	724.9 ± 13.6	6.84 ± 0.72	0.9994	2.43	1026	
Ellagic acid	0.512 ± 0.017	4.44 ± 1.15	0.9987	1196.0 ± 37	4.77 ± 1.28	0.9989	4.01	0,000428	
Ascorbic Acid	354.3 ± 13.7	3.72 ± 0.99	0.9989	339.7 ± 8.0	2.95 ± 0.42	0.9992	1.14	1043	

<sup>a</sup> ABTS discolouration ability of the antioxidant in relation to the ABTS discolouration ability of Trolox.

<sup>b</sup> ABTS discolouration ability of the antioxidant at pH 4.5 in relation to the ABTS discolouration ability of the antioxidant at pH 7.4.

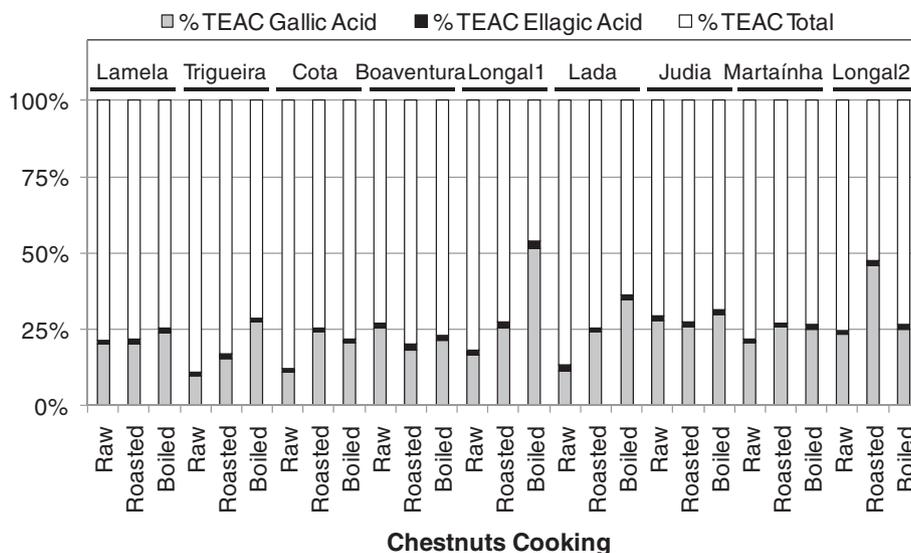


Fig. 4. Contribution of gallic and ellagic acid to the measured hydrophilic antioxidant activity of raw, roasted and boiled chestnuts.

increase with the cooking processes (13.5, 14.7 and 11.0 mg gallic acid/kg d.w. for the raw, roasted and boiled chestnuts, respectively), and so the compensation for the antioxidant activity due to the loss of vitamin C during the cooking process by the gallic acid, as observed for the Lada and Cota cultivars, is not observed for this cultivar. Another examples of the importance of gallic acid to the observed antioxidant activity of some of the chestnut cultivars after cooking are the Longal1 boiled and Longal2 roasted chestnuts. For the Longal1 cultivar, the gallic acid contributes to nearly 52% of the observed antioxidant activity of this cooked chestnut. For this cultivar the boiling process did not decrease significantly the vitamin C content, and although not significantly different from the raw chestnut, the antioxidant activity of the boiled cultivar is in average higher. This increase is due to an increase in the gallic acid content of the boiled chestnut from this cultivar (5.0 to 15.5 mg/kg d.w., respectively). Also for the roasted Longal2 cultivar, gallic acid contributes to 46% of the observed antioxidant activity. For this cultivar after roasting there is observed a decrease in the antioxidant activity compared to the raw chestnut (14%), and this decrease is followed by a larger decrease in the vitamin C content of this cultivar (26%), but by an increase of the gallic acid content (12.7 and 22.5 mg/kg d.w., for the raw and roasted chestnuts, respectively).

#### 4. Conclusions

The cooking process, either via boiling or roasting, changed significantly the vitamin C content and antioxidant activity of chestnuts. Although losses of Vitamin C of the chestnuts between 25% to 54% for the boiling process and 2–77% for the roasting process were observed, boiled and roasted chestnuts can still be good sources of vitamin C since it may represent 22.4% and 16.2% and 26.8% and 19.4%, respectively, of the recommended dietary intake for adult man and woman.

For the raw chestnuts the variation in vitamin C content of the chestnuts explains 99% of the antioxidant activity variation but for the roasted and boiled chestnuts this percentage significantly decreases to 51% and 88%, respectively. Although a high antioxidant activity is still present in the cooked chestnuts, the cause for this antioxidant activity is less dependent on the vitamin C content of the chestnuts. These results show that although for the raw chestnuts the vitamin C content of the chestnuts is the most important

antioxidant present in the chestnuts, during cooking besides vitamin C, also the gallic acid, presumably transferred from the peels to the fruit, can contribute significantly to the observed antioxidant activity.

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