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Primary and Secondary Metabolite Composition of Kernels from Three Cultivars of Portuguese Chestnut (*Castanea sativa* Mill.) at Different Stages of Industrial Transformation

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Chestnut (*Castanea sativa*) is an important basic food in rural diets and a major starch crop used in a similar way to potatoes. Chestnuts are a fundamental economic resource in the "chestnut regions" not only for the fruit but also for the chestnut wood. Chestnuts have become increasingly important with respect to human health, for example, as an alternative gluten-free flour source. Chestnuts are also a rich source of other beneficial compounds, but there have been few studies on the composition during processing. In this study, we analyzed the chemical composition of three Portuguese cultivars at different stages of industrial processing. The chestnut cultivars were Longal, Judia, and Martaínha. All three cultivars had high moisture contents but were low in ash, crude fat, and crude protein contents, with high starch and low fiber contents. The free amino acid contents, including various essential amino acids, varied depending on the cultivar. All three cultivars also had a significant content of polyphenolics with gallic acid; ellagic acid was predominant among hydrolyzable and condensed tannins. Many of these compounds are known to exert significant positive effects on human health. The one-way analysis of variance for fresh chestnut shows significant differences among the three cultivars for most of the studied parameters. The same statistical analysis applied to each one of the two cultivars (Judia and Longal) sampled for the four processing steps analyzed indicates a significant effect of this factor in practically all of the constituents. On the other hand, the two-way analysis of variance shows that, besides the residual, the processing step and the interaction cultivar × processing step were the factors that more contributed for the total variation observed in the constituents analyzed, while the contribution of cultivar was much less significant.

KEYWORDS: ***Castanea sativa*; fat; protein; starch; amino acids; fiber; ash; phenolics; gallic acid; ellagic acid; HPLC**

INTRODUCTION

Since early times chestnut has been an important economic resource in Europe and more recently in Asia and America, also playing an important environmental role in many agroforestry systems, particularly at present (*1*). During some historical periods, in various regions of Europe, the cultivation of chestnut became so dominant and indispensable for the survival of rural populations that some authors do not hesitate to identify these cultures as "chestnut civilizations" (*2*). *Castanea* genera belong to the Fagaceae family, which includes other ecologically and economically important tree species such as *Aesculus hippocastanum* (horse chestnut), *Betula pendula* (birch), *Fagus sylvatica* (beech), and *Quercus* species (oaks) (*3*). In Portugal, the most commonly cultivated chestnut species is *Castanea sativa* Mill. The main importers of the Portuguese chestnuts are, in order of priority, Spain, Italy, France, Brazil, the United Kingdom, and Switzerland; the first four countries are responsible for 91.5% of the exports of this nut from Portugal. In the past few years, the demand for Portuguese chestnuts in the United States has increased, but the import levels have not reached those of the major importers listed above (*4*). In former times, chestnuts were used as feedstuffs and as a substitute for potatoes and wheat flour. Nowadays, apart from boiling/baking and roasting, the utilization of chestnuts in Europe is for marron glace (*5*).

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Chestnuts are found in three major geographical areas: Asia, where *Castanea crenata* Sieb. and Zucc. (in Japan), *Castanea molíssima* Bl. (in China and Korea), *Castanea seguinii* Dode, *Castanea davidii* Dode, *Castanea henryi* Rehder, and Wilson (Skan) (in China) thrive; North America, where *Castanea dentata* Borkh., *Castanea pumila* (L.) Mill., *Castanea floridana* Ashe (Sarg.), *Castanea ashei* (Sudw.) Ashe, *Castanea alnifolia* Nutt., and *Castanea paucispina* Ash. predominate; and Europe, where the sweet chestnut (*C. sativa* Mill.) is predominant (*1*).

Previous studies on the chemical composition of chestnut kernels focused on starch, neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), fat, crude protein, ash, and minerals contents (*6*, *7*). Other researchers analyzed the lipids and fatty acid composition of chestnut kernels (*8*, *9*). There have been some published studies on the free amino acids content of chestnuts (*10*, *11*). There have been several studies on the phenolic composition of chestnut wood and leaves but very few studies on kernels (*12*), and primarily for single components, e.g., the procyanidins (*13*−*15*).

The aims of this work were to analyze the composition of health-related components of chestnut kernels at different stages of industrial transformation for both primary and secondary metabolites. This data will then be used as part of ongoing research for improving processing methods and selection of *C. sativa* cultivars for breeding programs and optimizing health benefits of chestnuts.

MATERIALS AND METHODS

Plant Material. The cultivars analyzed were Longal, Judia, and Martaínha; these were harvested in October/November 2005 from orchards around Bragança, North East Portugal. The samples were collected at Sortegel-Produtos Congelados S.A., an enterprise involved in processing chestnut kernels and commercialization of this fruit, as both fresh (19%) and frozen (74%) kernels. Because of poor kernel yields during the harvest year, the cultivar Martaínha was not industrially processed, and thus, samples at stages B−D were not available. The samples were collected at the end of each processing step: (A) fresh, (B) after storage for 2 months at ±0 °C, (C) after industrial steam peeling at Sortegel, and (D) after freezing with liquid air and −20 °C storage at Sortegel (**Figure 1**). After the collection of each sample of the three cultivars at the respective processing stage, six subsamples were taken and stored in a refrigerator at ±2 °C (for samples from stages A−C), for a maximum of 3 days until they were hand-peeled (stages A and B only). Samples from stage C were processed immediately, and samples from stage D were kept at −20 °C until analyzed. The samples were processed either by air-drying or freeze-drying depending on the analyses to be performed.

Chemicals. All chemicals and reagents were of analytical grade and were obtained from various commercial sources (Sigma/Aldrich, Merck, and Pronalab). All solvents were of high-performance liquid chromatography (HPLC) grade, and all water was ultrapure. All amino acid and phenolic standards were obtained from Sigma-Aldrich. For all standards, HPLC calibration curves were constructed by injection of 20 μL of different stock concentrations of the standards.

Processing Samples for the Different Analyses. Samples were stored unshelled at ±2 °C until analysis for a maximum of 3 days. The shells and pellicle were manually removed (stages A and B). The raw shelled kernel samples were broken into small pieces approximately 2 cm square, and a portion was dried in a model ULM/SLM 800 air-forced oven (Memmert, Schwabach, Germany), at 65 °C until constant weight (for at least 24 h), to determine dry matter and for use in various primary metabolite analyses (*8*). The remaining part, for the amino acids and phenolic analyses, was frozen with liquid nitrogen, powdered, and freeze-dried in a Dura Dry μP from F.T.S. Systems (Stone Ridge, New York) for 48 h. Air-dried samples were sequentially ground to homogeneous fine powders in a model D-7319 electric hammer-mill (Dietz-Motoren GmbH & Co. KG, Dettingen, Germany) followed by a model 843 food processor (Moulinex, Italy).

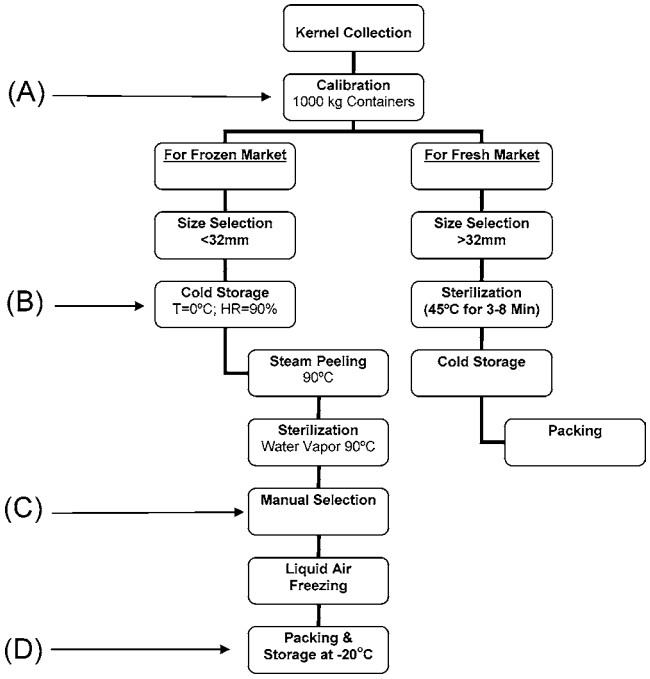

Figure 1. Schematic representation of the industrial processing steps for frozen and fresh chestnut kernels.

Dry Matter, Organic Matter, and Ash Contents. Subsamples of the air-dried samples (2.5 g) in duplicate for each sample were analyzed for residual water content and ash content using previously validated methods (*16*). In the first step, samples were submitted to a drying processing in a model USM/ULM 500 oven at 105 °C (Memmert, Schwabach, Germany) for 12 h, and then, the samples were weighed. Dried samples were incinerated in a Furnace model 6000 high-temperature oven (Thermolyne Corp., Dubuque, United States) for 3 h at 550 °C, and the ash content was obtained.

Extraction and Quantification of Crude Fat. For determination of crude fat contents, subsamples of the air-dried samples (3 g), in duplicate for each sample, were extracted with petroleum ether for 6 h in a Soxhlet apparatus (Italy, Lurano). The residue obtained by evaporation of the solvent in a rotary evaporator was weighed; the residue constituted the crude fat (*17*).

Extraction and Quantification of Crude Protein. Subsamples of the air-dried samples (1 g) in duplicate for each sample were analyzed for total nitrogen by the Kjeldahl method in combination with a selenium catalyst using a Büchi 435 digestion unit and a Büchi B-324 (Flawil, Switzerland) distillation unit (*17*). The crude protein content was calculated by using 5.3 as the conversion factor, according to McCarthy and Meredith (*18*).

Extraction and Quantification of Starch. Subsamples of the air-dried samples (0.05 g) in triplicate for each sample were analyzed for starch. The method used started with a conversion of the starch to glucose during two stages of an enzymatic treatment that was followed by a colorimetric determination of the glucose using a glucose-specific coupled enzyme reaction and chromogen system (*19*). The method involved the initial enzymatic treatment of the finely powdered plant material with α-amylase using a previously described method (*20*). During this processing step, the initial breakdown of the starch to dextrins and oligosaccharides occurred, ensuring a more effective quantitative conversion to glucose during a second incubation with amyloglucosidase. The colorimetric determination of glucose was done using the single-solution reagent method previously reported, which involved the coupled enzymatic glucose oxidase/peroxidase reaction in combination with the 4-amino antipyrine chromogen system (*21*, *22*).

Extraction and Quantification of Fiber (NDF, ADF, and ADL). The air-dried samples were weighed (1.0 g for NDF and 0.8 g for ADF and ADL) in duplicate, and the respective quantifications of NDF, ADF, and ADL were determined by the Van Soest detergent system (*23*).

Because of the starchy nature of samples, α-amylase, a thermoresistant enzyme (Sigma Chemical Co., Spruce Street, St. Louis, MO), was also used to enzymatically transform the chains of amylose and amylopectin to make them more susceptible to extraction from the fiber fraction.

Extraction and HPLC Analysis of Free Amino Acids. The freeze-dried samples were weighed in triplicate (0.2 g) into 15 mL glass centrifuge tubes. Extraction was performed by sequential extraction with first 70% and then 90% v/v methanol in combination with a heating plate and a homogenizer. After each extraction, the samples were centrifuged. The purification of the extracts was done using a previously described method (*24*); essentially, samples were loaded into plastic columns (Chromabond of Macherey-Nagel) containing 1 mL of cationic ion-change resin (Dowex (H^+) 50WX8-400, Sigma) connected to a solid-phase extraction unit (Varian Vac Elut SPS 24). The final eluates obtained were evaporated on a heating plate at 35 °C with an air flow vacuum pump, and the evaporated fractions were resuspended with 300 μL of ultrapure water and then filtered (Whatman general purpose 0.2 μm filter) and stored in vials in the refrigerator until HPLC analysis. The analysis was performed using a 150 mm × 4.6 mm, 3 μm C18 Spherisorb S3 ODS2 reverse-phase column (Waters, United States) in combination with a Gilson HPLC system (Wisconsin) consisting of a model 118 mixing chamber, model 402 high-pressure pump, model 231 XL detector, and a Jones Chromatography (Grace Vydac/Jones Chromatography, Ontario, Canada) thermostatically controlled oven for the column set at 30 °C. Data were processed using the Uni-Point software. The solvents were A [350 mM disodium hydrogen phosphate with 250 mM propionic acid (1:1), acetonitrile, and ultrapure water, 40:8:52] and B (acetonitrile, methanol, and ultrapure water, 30:30:40); both solvents were filtered (0.2 μm) and degassed before use. The gradient and flow rates were as follows: 0 min, 100% A; 9.5 min, 89% A; 11.0 min, 88% A; 13.6 min, 80% A; 20.4 min, 55% A; 23.4 min, 50% A at 1.3 mL/min; then 25.4 min, 40% A; 32.0 min, 100% B at 0.8 mL/min; followed by 34.0−37.0 min, 100% A at 1.3 mL/min. A mixture of amino acid standards was freshly prepared and run with each set of samples.

Extraction and Quantification of Total Phenolics. Subsamples of the freeze-dried chestnut kernel samples (3 × 40 mg) were extracted with 1 mL of 70% methanol at 70 °C for 30 min in 2 mL screw-top microtubes, using a vortex mixer every 5 min to optimize extraction. The samples were centrifuged (17000*g*, 4 °C, 20 min), and subsamples of the supernatant (3 × 100 μL) were used for determining total phenolics contents following the Folin−Ciocalteau spectrophotometer method. Gallic acid was used to produce the calibration curve, using a previously described method with a minor modification in that sample extracts and the gallic acid standard volumes used in the assay were 100 μL instead of 50 μL (*25*). Gallic acid calibration curves were produced each week.

Extraction and HPLC Analysis and Quantification of Free Gallic and Ellagic Acids. Subsamples of the freeze-dried chestnut kernel samples (3 × 40 mg) were extracted, using 950 μL of 70% methanol with 50 μL of 1 mg/mL naringin (in 100% methanol) as the extraction standard, at 70 °C for 30 min in 2 mL screw-top microtubes. The tubes were vortex mixed every 5 min to optimize extraction. The samples were centrifuged (17000*g*, 4 °C, 20 min) and filtered (0.2 μm general purpose filter), and HPLC analyses were performed using methods previously described (*26*); A 250 mm × 4.6 mm, 5 μm Luna C_{18} (*2*) main column, with a Securityguard precolumn (Phenomenex, United Kingdom) with a C_{18} cartridge in combination with a Thermo-Finnigan Surveyor HPLC system (solvent degasser, quaternary pump, thermostatically controlled autosampler set at 10 °C, thermostatically controlled column oven set at 25 °C, a photodiode array detector set to collect overall data from 200-600 nm, and selected wavelengths of 227, 270, 370, and 520 nm). Peak identifications were confirmed from retention times, UV spectroscopic data, and direct comparison to pure standards.

Statistical Analysis. To compare the three cultivars (Judia, Longal, and Martaínha) sampled for the processing step A (fresh) and the two cultivars (Judia and Longal) sampled for all of the processing steps evaluated (A, fresh; B, after storage; C, after industrial steam peeling; and D, after freezing), data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test with a 0.05 significance level. Additionally, for these two cultivars, data were analyzed by two-way ANOVA. In this case, cultivars and processing steps were the sources of variation considered, having as the error term the fixed effect of these factors. The contribution of each one of the sources (cultivar, processing step, and their interaction cultivar × processing step) for the total variation observed was measured as described by some authors (*27*, *28*).

Table 1. Basic Chemical Composition (g/100 g DW) and Phenolics (mg/g FW) of Fresh (Processing Step A) Raw Shelled Chestnut Kernels from Three Cultivars[a]

parameters	Judia	Longal	Martaínha	significance level
dry matter[b]	50.37 ± 1.54 ab	53.87 ± 3.83 b	48.73 ± 3.09 a	*
crude fat	1.72 ± 0.39	1.56 ± 0.31	1.89 ± 0.51	NS
crude protein	4.87 ± 0.33 b	5.13 ± 0.43 b	3.89 ± 0.13 a	***
starch	64.86 ± 1.63	64.15 ± 3.50	64.82 ± 1.33	NS
		fiber		
NDF	13.18 ± 2.65	13.75 ± 2.31	13.11 ± 1.05	NS
ADF	2.68 ± 0.27	2.54 ± 0.22	2.54 ± 0.37	NS
ADL	0.29 ± 0.21	0.22 ± 0.17	0.21 ± 0.10	NS
cellulose	2.40 ± 0.27	2.32 ± 0.29	2.33 ± 0.42	NS
total ashes	2.34 ± 0.20 b	1.91 ± 0.05 a	1.87 ± 0.20 a	***
total phenolics[c]	21.10 ± 5.11 b	15.80 ± 5.69 a	22.69 ± 8.39 b	**
gallic acid[d]	4.30 ± 1.52 a	3.46 ± 1.72 a	9.07 ± 3.43 b	***
free ellagic acid[d]	4.84 ± 1.37 b	2.71 ± 0.70 a	9.64 ± 3.39 c	***

[a] Tabulated values are sample means ± standard deviations (SDs) of the mean. NS, not significant ($P > 0.05$); *, **, and ***, significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Within each row, means with a different letter are significantly different. [b] Data are presented as means ± SD g/100 g FW. [c] Data are presented as means ± SD mg gallic acid equivalents/gram fresh weight. [d] Data are presented as means ± SD mg phenolic/gram fresh weight.

RESULTS AND DISCUSSION

For each of the different analyses, both one-way and two-way ANOVAs were performed. There were significant differences detected both for the comparison of the three cultivars Judia, Longal, and Martaínha at the processing stage A and for the two cultivars Longal and Judia at all processing stages (A−D).

Previous studies have shown that the moisture content of chestnut kernels was between 40.3 and 60.1% of fresh weight (*7*). The results of the current research show that all three cultivars had high moisture contents but low ash contents (**Tables 1** and **2**). The values for moisture (dry matter) were 48.7, 53.9, and 50.4%, and the values for ash contents were 1.9, 1.9, and 2.3 of dry matter in fresh kernels of Martaínha, Longal, and Judia, respectively, in processing stage A. For the remaining processing steps, the highest values found for ash content in the cultivars Longal and Judia were, respectively, 2.3 and 2.1% of dry matter (**Table 2**). There was a significant difference in the ash contents of the three cultivars, probably due to differences in cultivation conditions (**Table 1**).

The values for crude fat content previously reported for chestnut kernels were between 1.7 and 4.0% of dry matter (*7*). This study indicates for the cultivar Longal grown in Galicia a crude fat content of 2.4% DM, which is similar to the values found in our Portuguese Longal. The current results revealed that all three cultivars had low crude fat contents (**Table 1**) of 1.9, 1.6, and 1.7% of dry matter in fresh kernels of Martaínha, Longal, and Judia, respectively, in processing stage A (**Table 1**); there were no significant differences in crude fat contents among the three cultivars. For the remaining processing steps, the highest value found for crude fat was 2.6% of dry matter for both Longal and Judia cultivars in the processing step C (**Table 2**). Recent analyses of 17 sweet chestnut cultivars

Table 2. Basic Chemical Composition (g/100 g DM) and Phenolics (mg/g FW) of Raw Shelled Chestnut Kernels from Two Cultivars on the Four Processing Steps Analyzed[a]

parameters	A	B	C	D	SL
			Judia		
dry matter[b]	50.37 ± 1.54 ab	54.57 ± 1.28 c	51.30 ± 2.07 b	49.24 ± 0.41 a	***
crude fat	1.72 ± 0.39 a	1.94 ± 0.70 a	2.58 ± 0.16 b	2.47 ± 0.07 b	**
crude protein ($N \times 5.30$)	4.87 ± 0.33 a	5.21 ± 0.20 b	5.13 ± 0.20 ab	5.20 ± 0.25 b	NS*
starch	64.86 ± 1.63 b	58.37 ± 2.23 a	62.07 ± 1.82 b	57.54 ± 3.62 a	***
fiber					
NDF	13.18 ± 2.65 a	13.31 ± 1.38 a	12.67 ± 2.48 a	17.50 ± 0.92 b	**
ADF	2.68 ± 0.27 a	2.55 ± 0.28 a	3.47 ± 0.73 b	3.25 ± 0.32 b	**
ADL	0.29 ± 0.21	0.29 ± 0.10	0.37 ± 0.20	0.31 ± 0.16	NS
cellulose	2.40 ± 0.27 a	2.28 ± 0.24 a	3.11 ± 0.60 b	2.93 ± 0.45 b	**
total ashes	2.34 ± 0.20 c	2.09 ± 0.05 b	1.81 ± 0.08 a	2.10 ± 0.11 b	***
total phenolics[c]	21.10 ± 5.11 a	29.87 ± 10.54 b	28.11 ± 4.54 b	36.73 ± 4.78 c	***
gallic acid[d]	4.30 ± 1.52 b	2.76 ± 1.66 a	5.74 ± 2.77 c	3.98 ± 1.49 ab	***
free ellagic acid[d]	4.84 ± 1.37 a	8.99 ± 1.03 c	7.14 ± 1.48 b	7.45 ± 0.97 b	***
			Longal		
dry matter[b]	53.87 ± 3.83 b	49.32 ± 0.90 a	51.10 ± 0.85 a	49.51 ± 1.42 a	**
crude fat	1.56 ± 0.31 a	1.56 ± 0.57 a	2.58 ± 0.13 b	2.35 ± 0.28 b	***
crude protein ($N \times 5.30$)	5.13 ± 0.43 a	5.48 ± 0.11 b	5.03 ± 0.12 a	5.06 ± 0.21 a	*
starch	64.15 ± 3.50 b	61.05 ± 2.37 a	65.36 ± 2.05 b	59.31 ± 1.36 a	**
fiber					
NDF	13.75 ± 2.31 a	14.21 ± 1.86 a	12.61 ± 1.44 a	16.81 ± 0.63 b	**
ADF	2.54 ± 0.22 a	2.60 ± 0.36 a	3.59 ± 0.24 c	3.09 ± 0.41 b	***
ADL	0.22 ± 0.17 a	0.18 ± 0.06 a	0.49 ± 0.23 b	0.18 ± 0.11 a	**
cellulose	2.32 ± 0.29 a	2.43 ± 0.33 a	3.11 ± 0.19 b	2.91 ± 0.36 b	***
total ashes	1.91 ± 0.05 a	2.34 ± 0.06 b	1.98 ± 0.29 a	1.97 ± 0.04 a	***
total phenolics[c]	15.80 ± 5.69 a	29.29 ± 3.96 b	34.55 ± 7.51 c	29.96 ± 4.12 b	**
gallic acid[d]	3.46 ± 1.72	4.06 ± 2.58	4.69 ± 2.84	5.12 ± 2.49	NS
free ellagic acid[d]	2.71 ± 0.68 a	10.52 ± 2.99 c	6.97 ± 1.28 b	7.93 ± 1.15 b	***

[a] Tabulated values are sample means ± standard deviations (SD) of the mean. NS, not significant ($P > 0.05$); NS*, tendentiously significant ($0.05 < P < 0.10$); *, **, and ***, significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Within each row and for each one of the cultivars analyzed, means with a different letter are significantly different. [b] Data are presented as means ± SD g/100 g FW. [c] Data are presented as means ± SD mg gallic acid equivalents/gram fresh weight. [d] Data are presented as means ± SD mg phenolic/gram fresh weight.

showed values for crude fat from 1.67 to 3.50% of dry matter, lowest in cv. Lada (PDO Padrela) and highest in cv. Aveleira (PDO Terra Fria), respectively (*9*). Chestnut crude fat is predominated by three unsaturated fatty acids, oleic (C18:1), linoleic (C18:2), and α-linolenic (C18:3) acids, which account for more than 85% of the total fatty acid content (*9*). Mono- and polyunsaturated fatty acids (MUFA and PUFA) have been associated with positive health effects. There are reviews giving dietary recommendations for *ω*-3 fatty acids, including α-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid, to achieve nutrient adequacy and to prevent and treat cardiovascular diseases (*29, 30*). The cardioprotective benefits of *ω*-3 fatty acids may be attributed to multiple physiological effects on lipids, blood pressure, vascular function, cardiac rhythms, platelet function, and inflammatory responses (*31*). In addition, there is data suggesting that both *ω*-3 and *ω*-6 PUFA have positive health effects in humans (*32*).

In a previous study, the cultivar Longal grown in Galicia had a value of 5.2% crude protein (*7*). The current analysis found that all three cultivars had low crude protein contents (**Table 1**) of 3.9, 5.1, and 4.9% of dry matter in fresh kernels of Martaínha, Longal, and Judia, respectively in processing stage A (**Table 1**). For the remaining processing steps, the highest values found for crude protein content in the cultivars Longal and Judia were, respectively, 5.5 and 5.2% of dry matter (**Table 2**). Previous studies have found that the crude protein content of chestnut kernels was between 4.5 and 9.6% of dry matter, using 5.3 as a multiplicative factor (*7*). There was a significant difference in crude protein contents of fresh kernels of the three cultivars, with Martaínha having the lowest content (**Table 1**).

Previous studies have shown that the starch content of chestnut kernels was between 42.2 and 66.6% of dry matter (*7*). The cultivar with the highest starch content was Longal grown in Galicia (*7*). The results of the current research show that all three cultivars had high starch contents of 64.8, 64.2, and 64.9% of dry matter in fresh kernels of Martaínha, Longal, and Judia, respectively, in processing stage A (**Table 1**). For the remaining processing steps, the highest values found for starch content in the cultivars Longal and Judia were, respectively, 65.4 and 62.1% of dry matter (**Table 2**). There was no significant difference in the total starch contents of the three cultivars (**Table 1**). However, there may be more subtle changes in branching structure and physicochemical properties that cannot be determined from the total starch assay used that occur in the later processing stages (*33*). It is known that the chestnut fruits store a reserve in the form of starch in cotyledons, and the content is 3−4-fold higher than found in other nuts (*34*). The average value for starch content found in this study is slightly higher than those obtained by others researchers (*7*) who reported 57% starch relative to dry weight and higher than the value obtained by a previous work (*6*) for the Portuguese cultivar Longal (53−55%).

The three main plant fiber fractions are generated by detergent and acid treatments (*35*). The first fraction is generated by treating the sample with a neutral detergent creating two fractions: cellular contents [neutral detergent solubles (NDS)] that include protein, sugars, starch, lipids, nonprotein nitrogen, pectin, fructosans, water-soluble minerals, and vitamins; and cell wall components, representing the total fibrous fraction, which consists of cellulose, hemicellulose, lignin, silica, keratin,

Table 3. Free Amino Acids Contents (Expressed as mg/100 g FW and DW in Parentheses) of Fresh (Processing Step A) Raw Shelled Chestnut Kernels from Three Cultivars[a]

amino acids	Judia	Longal	Martaínha	significance level
Tyr	T	T	T	
Trp	T	T	T	
Phe	3.45 ± 0.80 b	4.53 ± 1.03 c	1.90 ± 0.62 a	***
	(6.22 ± 1.44 b)	(9.19 ± 2.09 c)	(3.64 ± 1.19 a)	
Asp	76.40 ± 16.25 b	60.32 ± 14.74 a	52.63 ± 13.33 a	**
	(137.87 ± 29.33 b)	(122.45 ± 29.91 ab)	(100.65 ± 25.50 a)	
Glu	109.82 ± 22.59 b	72. 38 ± 15.24 a	96.55 ± 18.53 b	***
	(198.18 ± 40.76 b)	(146.93 ± 30.95 a)	(184.64 ± 35.44 b)	
Asn	149. 45 ± 34.93 b	140.54 ± 25.05 b	62.02 ± 13.49 a	***
	(269.70 ± 63.04 b	(285.30 ± 50.85 b)	(118.61 ± 25.79 a)	
Gln	16.46 ± 2.80 c	14.49 ± 2.32 b	9.18 ± 1.53 a	***
	(29.70 ± 5.06 b)	(29.43 ± 4.72 b)	(17.55 ± 2.93 a)	
Arg	30.01 ± 10.46 b	25.42 ± 8.65 b	4.54 ± 1.43 a	***
	(54.16 ± 18.87 b)	(51.61 ± 17.55 b)	(8.67 ± 2.74 a)	
Ser	7.10 ± 1.99 c	5.63 ± 1.39 b	2.69 ± 0.56 a	***
	(12.81 ± 3.59 b)	(11.44 ± 2.83 b)	(5.14 ± 1.08 a)	
Gly	T	T	T	
Thr	T	T	T	
Ala	13. 55 ± 3.46 a	35.37 ± 8.54 b	11.60 ± 1.92 a	***
	(24.45 ± 6.25 a)	(71.80 ± 17.34 b)	(22.18 ± 3.66 a)	
Val	7.16 ± 1.35 b	6.70 ± 1.23 b	5.23 ± 1.22 a	**
	(12.92 ± 2.44 b)	(13.60 ± 2.51 b)	(10.00 ± 2.34 a)	
Ile	5.19 ± 1.12 a	4.40 ± 1.06 a	14.84 ± 4.91 b	***
	(9.37 ± 2.02 a)	(8.94 ± 2.15 a)	(28.39 ± 9.39 b)	
Leu	2.25 ± 0.52 c	1.50 ± 0.43 b	0.79 ± 0.37 a	***
	(4.05 ± 0.95 c)	(3.05 ± 0.87 b)	(1.52 ± 0.71 a)	
Totals	420.84 (759.43)	371.28 (753.74)	261.97 (500.99)	

[a] T, trace (detected in some samples but levels too low to be accurately quantified). Essential amino acids are highlighted in bold text. NS, not significant ($P > 0.05$); *, **, and ***, significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Within each row, means with a different letter are significantly different.

waxes, cutin, insoluble minerals, and lignified nitrogen compounds and are expressed as NDF (*35*). Treatment of the sample with acid detergent produces two fractions: All of the NDS components and also hemicellulose (the only compound of the cell wall components that is soluble in acid detergent) and the acid insoluble material (the rest of the cell wall components) that is expressed as ADF (*35*). The ADF fraction is subsequently treated with 72% v/v H_2SO_4 to remove the cellulose, and the solid residue remaining is the crude lignin that is expressed as ADL (*35*). The values for NDF and ADF contents found in previous chestnut kernel analyses were between 9.4 and 28.5 and 2.3−4.5% of dry matter, respectively (*7*). In this study, the cultivar Longal grown in Galicia had 12.1% DM for NDF and had the lowest value for ADF of 2.3% of dry matter. The current results revealed that all three cultivars had relatively low NDF, ADF, and ADL contents of 13.1, 13.8, and 13.2%; 2.5, 2.5, and 2.7%; and 0.2, 0.2, and 0.3% of dry matter in fresh kernels of Martaínha, Longal, and Judia, respectively, in processing stage A (**Table 1**). For the remaining processing steps, the highest values found for fiber content (NDF, ADF, and ADL) in the cultivars Longal and Judia were, respectively, 16.8 and 17.5%, 3.6 and 3.5%, and 0.5 and 0.4% of dry matter (**Table 2)**. There were no significant differences for each fiber fraction among the three cultivars (**Table 1**).

Free amino acids in plant foods are generally divided into two classes, essential and nonessential. Essential amino acids are those that cannot be synthesized in vivo by humans; therefore, plants (fruits, nuts, vegetables, etc.) are an important source of these essential amino acids. The essential amino acids found in *C. sativa* kernels were arginine, isoleucine, leucine, threonine, valine, phenylalanine, and tryptophan (**Tables 3** and **4**). In total, 15 amino acids were present in the chestnut cultivars studied, aspartic acid, glutamic acid, asparagine, serine, glutamine, glycine, threonine, arginine, alanine, tyrosine, valine, tryptophan, isoleucine, leucine, and phenylalanine; however, some of them (Tyr, Trp, Gly, and Thr) were only present in trace amounts (**Tables 3** and **4**). Trace values of amino acids mean that a peak was detected but was too small to be quantified. In the current study, the highest values found for each of the free amino acids (mg/100 g FW) in Martaínha, Judia, and Longal, respectively, were for processing step A: aspartic acid (52.6, 76.4, and 60.3); glutamic acid (96.6, 109.8, and 72.4); asparagine (62.0, 149.5, and 140.5); serine (2.7, 7.1, and 5.6); glutamine (9.2, 16.5, and 14.5); glycine (trace); threonine (trace); arginine (4.5, 30.0, and 25.4); alanine (11.6, 13.5, and 35.4); tyrosine (trace); valine (5.2, 7.2, and 6.7); tryptophan (trace); isoleucine (14.8, 5.2, and 4.4); leucine (0.8, 2.3, and 1.5); and phenylalanine (1.9, 3.5, and 4.5) (**Table 3**). The values found for the cultivars Judia and Longal, respectively, were for processing step B: aspartic acid (43.8 and 38.3); glutamic acid (79.8 and 101.9); asparagine (90.2 and 121.0); serine (4.4 and 6.4); glutamine (9.9 and 8.4); glycine (trace); threonine (trace); arginine (30.0 and 48.2); alanine (10.1 and 15.0); tyrosine (trace); valine (4.9 and 11.4); tryptophan (trace); isoleucine (7.1 and 6.7); leucine (2.9 only for Longal); and phenylalanine (1.3 and 5.4) (**Table 4**). The values found for the cultivars Judia and Longal, respectively, were for processing step C: aspartic acid (22.1 and 13.5); glutamic acid (51.8 and 27.4); asparagine (173.1 and 105.1); serine (9.4 and 4.8); glutamine (13.8 and 6.9); glycine (2.1 only for Judia); threonine (2.9 only for Judia); arginine (48.6 and 37.7); alanine (45.6 and 26.9); tyrosine (1.4 only for Judia); valine (11.5 and 14.1); tryptophan (trace); isoleucine (8.9 and 9.9); leucine (7.3 and 5.8); and phenylalanine (9.6 and 5.2) (**Table 4**). The values found for the cultivars Judia and Longal, respectively, were for processing step D: aspartic acid (41.1

Table 4. Free Amino Acids Contents (Expressed as mg/100 g FW and DW in Parentheses) of Raw Shelled Chestnut Kernels from Two Cultivars on the Four Processing Steps Analyzed[a]

amino acids	A	B	C	D	SL
			Judia		
Tyr	T	T	1.36 ± 0.27	T	
			(2.69 ± 0.53)		
Trp	T	T	T	T	
Phe	3.45 ± 0.80 b	1.33 ± 0.39 a	9.62 ± 1.41 d	6.49 ± 1.90 c	***
	(6.22 ± 1.44) b	(2.41 ± 0.71) a	(18.94 ± 2.78) d	(13.24 ± 3.88) c	
Asp	76.40 ± 16.25 c	43.78 ± 12.65 b	22.08 ± 3.12 a	41.14 ± 10.30 b	***
	(137.87 ± 29.33) b	(79.01 ± 22.83) b	(43.46 ± 6.15) a	(83.98 ± 21.03) b	
Glu	109.82 ± 22.59 c	79.83 ± 15.69 b	51.79 ± 6.40 a	67.37 ± 18.69 b	***
	(198.18 ± 40.76) b	(144.06 ± 28.32) b	(101.94 ± 12.59) a	(137.52 ± 38.15) b	
Asn	149.45 ± 34.93 c	90.18 ± 14.99 a	173.14 ± 27.40 d	119.43 ± 27.64 b	***
	(269.70 ± 63.04) b	(162.74 ± 27.05) a	(340.83 ± 53.93) c	(243.80 ± 56.42) b	
Gln	16.46 ± 2.80 c	9.86 ± 1.50 a	13.83 ± 2.64 b	16.63 ± 3.03 c	***
	(29.70 ± 5.06) b	(17.79 ± 2.71) a	(27.23 ± 5.19) b	(33.94 ± 6.19) c	
Arg	30.01 ± 10.46 a	30.04 ± 3.99 a	48.55 ± 8.14 b	43.29 ± 8.44 b	***
	(54.16 ± 18.87) a	(54.21 ± 7.20) a	(95.57 ± 16.01) b	(88.37 ± 17.23) b	
Ser	7.10 ± 1.99 b	4.35 ± 0.85 a	9.35 ± 1.44 c	4.84 ± 1.37 a	***
	(12.81 ± 3.59) b	(7.86 ± 1.53) a	(18.40 ± 2.83) c	(9.88 ± 2.79) a	
Gly	T	T	2.13 ± 0.59	T	
			(4.18 ± 1.15)		
Thre	T	T	2.87 ± 0.59	T	
			(5.65 ± 1.16)		
Ala	13.55 ± 3.46 b	10.11 ± 2.35 a	45.59 ± 7.95 c	14.40 ± 3.38 b	***
	(24.45 ± 6.25) ab	(18.25 ± 4.25) a	(89.75 ± 15.65) c	(29.39 ± 6.91) b	
Val	7.16 ± 1.35 b	4.91 ± 1.07 a	11.54 ± 1.26 c	11.46 ± 3.03 c	***
	(12.92 ± 2.44) b	(8.87 ± 1.92) a	(22.73 ± 2.48) c	(23.39 ± 6.18) c	
Ile	5.19 ± 1.12 a	7.13 ± 3.87 ab	8.89 ± 1.13 b	8.15 ± 3.16 b	**
	(9.37 ± 2.02) a	(12.87 ± 6.99) ab	(17.51 ± 2.23) c	(16.63 ± 6.46) bc	***
Leu	2.25 ± 0.52 a	T	7.34 ± 1.54 b	6.65 ± 1.47 b	***
	(4.05 ± 0.95) a		(14.46 ± 3.04) b	(13.58 ± 3.00) b	
total	420.84	281.52	408.08	339.49	
	(759.4)	(508.1)	(803.3)	(693.7)	
			Longal		
Tyr	T	T	T	T	
Trp	T	T	T	T	
Phe	4.53 ± 1.03 ab	5.40 ± 0.84 c	5.15 ± 1.15 bc	4.14 ± 0.68 a	**
	(9.19 ± 2.09) a	(10.95 ± 1.70) b	(9.69 ± 2.17) ab	(8.37 ± 1.38) a	*
Asp	60.32 ± 14.74 c	38.34 ± 9.82 b	13.54 ± 2.82 a	33.69 ± 6.67 b	***
	(122.45 ± 29.91) c	(77.83 ± 19.94) b	(25.49 ± 5.32) a	(68.02 ± 13.47) b	
Glu	72.38 ± 15.24 b	101.87 ± 24.19 c	27.36 ± 5.65 a	60.33 ± 9.85 b	***
	(146.93 ± 30.95) b	(206.80 ± 49.10) c	(51.49 ± 10.64) a	(121.82 ± 19.89) b	
Asn	140.54 ± 25.05 c	121.04 ± 29.78 bc	105.05 ± 25.43 b	82.57 ± 18.97 a	***
	(285.30 ± 50.85) b	(245.73 ± 60.46) b	(197.74 ± 47.87) a	(166.72 ± 38.30) a	
Gln	14.49 ± 2.32 d	8.41 ± 1.43 b	6.85 ± 1.74 a	11.56 ± 2.01 c	***
	(29.43 ± 4.72) d	(17.07 ± 2.90) b	(12.89 ± 3.27) a	(23.34 ± 4.05) c	
Arg	25.42 ± 8.65 a	48.18 ± 10.11 c	37.70 ± 4.89 b	28.65 ± 8.78 a	***
	(51.61 ± 17.55) a	(97.81 ± 20.53) c	(70.97 ± 9.21) b	(57.84 ± 17.72) ab	
Ser	5.63 ± 1.39 bc	6.43 ± 2.01 c	4.76 ± 1.51 ab	4.12 ± 1.26 a	*
	(11.44 ± 2.83) bc	(13.05 ± 4.09) c	(8.96 ± 2.85) ab	(8.32 ± 2.54) a	**
Gly	T	T	T	T	
Thre	T	T	T	T	
Ala	35.37 ± 8.54 c	14.98 ± 5.26 a	26.87 ± 5.25 b	9.94 ± 4.11 a	***
	(71.80 ± 17.34) c	(30.42 ± 10.68) a	(50.59 ± 9.88) b	(20.07 ± 8.30) a	
Val	6.70 ± 1.23 a	11.42 ± 2.84 bc	14.12 ± 6.72 c	8.82 ± 2.06 ab	***
	(13.60 ± 2.51) a	(23.19 ± 5.77) bc	(26.59 ± 12.64) c	(17.82 ± 4.16) ab	**
Ile	4.40 ± 1.06 a	6.74 ± 1.48 a	9.93 ± 3.79 b	9.29 ± 4.29 b	***
	(8.94 ± 2.15) a	(13.68 ± 2.99) a	(18.70 ± 7.13) b	(18.75 ± 8.65) b	
Leu	1.50 ± 0.43 a	2.87 ± 0.72 b	5.79 ± 1.31 c	3.21 ± 0.75 b	***
	(3.05 ± 0.87) a	(5.83 ± 1.46) b	(10.89 ± 2.46) c	(6.48 ± 1.51) b	
total	371.28	365.68	257.12	256.32	
	(753.7)	(742.4)	(484.0)	(517.6)	

[a] T, trace (detected in some samples but levels too low to be accurately quantified). Essential amino acids are highlighted in bold text. NS, not significant ($P > 0.05$); *, **, and ***, significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Within each row and for each one of the cultivars analyzed, means with a different letter are significantly different.

and 33.7); glutamic acid (67.4 and 60.3); asparagine (119.4 and 82.6); serine (4.8 and 4.1); glutamine (16.6 and 11.6); glycine (trace); threonine (trace); arginine (43.3 and 28.7); alanine (14.4 and 9.9); tyrosine (trace); valine (11.5 and 8.8); tryptophan (trace); isoleucine (8.2 and 9.3); leucine (6.7 and 3.2); and phenylalanine (6.5 and 4.1) (**Table 4**).

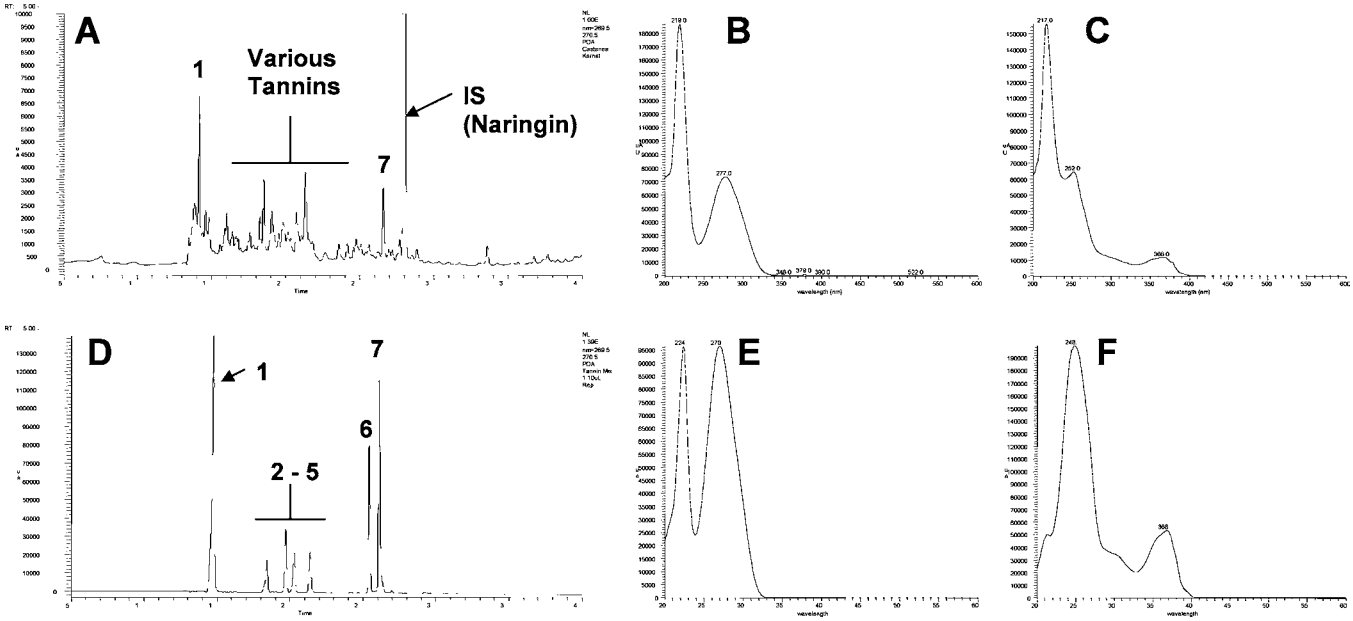

Figure 2. Chromatograms and UV–visible spectra of an example 20 μL injection of a Castanea kernel phenolic extract (**A**) and a 10 μL injection of a 0.1 mg/mL phenolic standard mixture (**D**). Spectra for gallic acid and ellagic acid in the samples (**B** and **C**, respectively) and in the standard mixture (**E** and **F**, respectively). Peak ID: 1, gallic acid; 2, procyanidin B1; 3, catechin; 4, procyanidin B2; 5, epicatechin; 6, rutin (quercetin 3-O-rutinoside); and 7, ellagic acid. There was a shift in the RT of the gallic acid in the sample as confirmed by injection of a gallic acid spiked sample, probably due to the larger injection volume of the 70% methanol extract.

Some studies analyzing the changes in the free amino acid composition of ripening chestnut seeds (*10*) at the maximum stage of maturity (mg/100 g FW) revealed the following values in the cultivars Dorée de Lyon and Sauvage des Cars, respectively: aspartic acid (44.0 and 35.0); glutamic acid (6.0 and 25.0); asparagine (120.0 and 74.0); serine (13.0 and 12.0); glutamine (5.0 and 8.0); glycine (8.0 and 9.0); threonine (9.0 and 11.0); arginine (4.0 and 30.0); alanine (48.0 and 67.0); tyrosine (4.0 and 3.0); valine (15.0 and 15.0); tryptophan (4.0 and 11.0); isoleucine (4.0 and 5.0); leucine (6.0 and 4.0); and phenylalanine (19.0 and 8.0). Other authors (*11*) studied the composition of free amino acids in several French chestnut cultivars, and the ranges of values (mg/100 g FW) found were as follows: aspartic acid (9.24−70.7); glutamic acid (3.05−81.36); asparagine (36.39−234.0); arginine (1.85−87.2); and alanine (22.45−78.99).

There were significant differences in individual amino acids in the fresh kernels of the three cultivars including some of the essential amino acids; kernels of Martaínha had a low content of the essential amino acids leucine arginine and valine as compared with the other two cultivars (**Tables 3** and **4**).

In the current study, the values of total phenolics were 22.7, 15.8, and 21.1 mg/g of fresh weight in kernels of Martaínha, Longal, and Judia, respectively, in processing stage A (**Table 1**). For the remaining processing steps, the highest values found for total phenolics content in the cultivars Longal and Judia were, respectively, 34.6 and 36.7 (mg/g of fresh weight) (**Table 2).** Previous studies have found a total phenolic content in leaves of chestnut of 147 mg/g dry weight, which equates to approximately 54 mg/g FW (*13*). Several species within the Fagaceae (including *Aesculus*, *Betula*, *Castanea*, *Castanopsis*, *Fagus*, and *Quercus*) have very high tannin contents in the wood, leaves, and often in the nuts.

Chestnut is known as a high tannin species and specifically in the wood and leaves of this species with both ellagitannins (*36*) and procyanidins (*15*) detected in various tissues. In the present study, free gallic acid and ellagic acid were identified and quantified; other tannins were present, but these were not identified (**Figure 2**). The gallic acid contents were 9.1, 3.5, and 4.3 mg/g FW for Martaínha, Longal, and Judia, respectively, in processing stage A (**Table 1**). For the remaining processing steps, the highest values found for free gallic acid content in the cultivars Longal and Judia were, respectively, 5.1 and 5.7 (mg/g of fresh weight) (**Table 2**).

Free gallic acid and ellagic acid are common in species such as grapes (*Vitis vinifera* L.), strawberries (*Fragaria* species), cranberries (*Vaccinium* species), and raspberries (*Rubus idaeus* L.), and high levels have been found in the fruit and seeds; these species also contain conjugated forms of ellagic acid, i.e., ellagitannins (*37*−*47*). Free gallic acid was present in all three *Castanea* cultivars (**Table 1**). Martaínha had the highest free gallic acid content (9.1 mg/g FW). Free ellagic acid (**Tables 1** and **2**) was found in the chestnut kernels, as well as various ellagitannins and procyanidins that were not determined in the present study. Previous studies on hardwood samples had found values of 19−89 mg/g of hardwood of total ellagic acid (*44*); European chestnut had the highest value of 89 mg/g total ellagic acid (*44*). Until this study, there was no data available for total phenolics or specific phenolics in chestnut kernels. In this current study, the values for free ellagic acid were 9.6, 2.7, and 4.8 mg/g of FW in fresh kernels of Martaínha, Longal, and Judia, respectively, in processing stage A (**Table 1**). For the remaining processing steps, the highest values found for free ellagic acid content in the cultivars Longal and Judia were, respectively, 10.5 and 9.0 (mg/g of FW) (**Table 2**). There is good evidence for the positive health effects of gallic and ellagic acids, especially their antioxidant activities, positive effects on cardiovascular functions, anticarcinogenic activities, and antiplasmodial activity (*37*, *39*, *40*, *42*, *45*−*50*).

The one-way ANOVA for fresh chestnut (processing step A) shows significant differences among the three cultivars for most of the studied parameters. Only the levels of crude fat, starch, and each of the three fiber fractions had no significant differences in variation (**Tables 1** and **3**).

The same statistical analysis applied to each one of the two cultivars sampled for the four processing steps indicates a significant effect of this factor in practically all of the constituents analyzed (**Tables 2** and **4**). In fact, only for gallic acid content in the case of cultivar Longal and for the ADL fiber fraction of cultivar Judia were no significant differences in the processing steps identified. For this last cultivar, the variation in the crude protein levels also was of low significance ($0.05 < P < 0.10$). Nevertheless, to confirm these results, as well as to explain with more security the effect of each one of the processing steps in the levels of the several constituents, these studies are being continued for the parameters reported in this paper and for further parameters associated with nutritional and structural properties of chestnut kernels.

Additionally, the two-way ANOVA (presented in the Supporting Information) shows that in the majority of parameters the contribution of the residual for the total variation observed was higher than the whole of the analyzed factors. What this means is that the detected differences were due primarily to the noncontrolled factors.

However, the contribution of the processing step with respect to the variation observed was important for a number of nutrients, namely, crude fat (26.5%), starch (23.9%), NDF (22.0%), ADF (26.2%), cellulose (22.1%), total phenolics (17.6%), free ellagic acid (35.2%), and the amino acids aspartic acid (46.7%), leucine (36.1%), glutamic acid (21.9%), glutamine (19.8%), alanine (18.2%), and isoleucine (13.9%). The interaction cultivar × processing step also had an interesting contribution to the variation observed in dry matter (30.9%), total ashes (36.9%), and in the amino acids phenylalanine (39.0%), glutamic acid (15.8%), asparagine (22.7%), arginine (28.4%), serine (27.7%), valine (15.0%), and mainly alanine (44.5%). On the other hand, the contribution of the cultivar for the total variation only had some significance in the amino acids glutamine (17.5%) and leucine (12.4%), while for the remaining amino acids, as well as for phenolics and all basic constituents, its contribution was in the majority of cases null or below 5%.

The results of the current study show that chestnut kernels contain significant concentrations of primary and secondary metabolites that are known to have positive health effects in humans. Chestnuts are an increasingly popular food, as fresh, frozen, roasted, and in other processed form such as marron glacé. However, for determinations of total compounds (fat, protein, starch, and fiber), there may be more subtle qualitative changes that cannot be measured by using these general assays; that is, it is known that cooking chestnuts affects the physicochemical properties of starch but not total starch content (*33*). Therefore, further studies need to be performed using more sensitive assays, e.g., (sodium dodecyl sulfate) polyacrylamide gel electrophoresis for proteins, liquid chromatography−mass spectrometry, and gas chromatography−mass spectrometry of lipids and fatty acids and enzymatic and NMR studies for starch and fiber. In addition, more qualitative and quantitative studies need to be done on the ellagitannins and procyanidins in chestnut kernels, especially since these compounds in other food plants have been shown to have positive health effects.

Supporting Information Available: Tables of statistical analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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