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Chestnut (*Castanea sativa* Mill.) Fruit Composition & Quality

Effects of Industrial Processing on Nutrients & Secondary Metabolites

Tese apresentada para o efeito de obtenção do grau de Doutor em Ciências Agrárias e Florestais, de acordo com o disposto no nº 2 do artigo 8 do Decreto-Lei nº388/70 (Decreto-Lei nº 216/92, de 13 de Outubro).

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Aos meus pais e irmã

Ao meu marido

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RESUMO

O castanheiro é cultivado um pouco por toda a Europa do Sul (principalmente Itália, Portugal, França e Espanha), Turquia, América do Norte, América do Sul (Chile e Bolívia), Ásia (China, Japão e Coreia), Austrália e Nova Zelândia, onde os seus frutos são consumidos em larga escala. Face ao seu conteúdo em amido, açúcares livres, fibra, proteínas, lípidos, vitaminas e sais minerais, bem como em outros compostos biologicamente activos, tais como polifenóis, reconhece-se que a castanha constitui uma importante fonte alimentar rica em compostos benéficos para a saúde. Contudo, o facto de existirem ainda muito poucos estudos sobre a composição química deste fruto e sua variação ao longo das diferentes fases de processamento industrial, constituiu o principal objectivo para a realização do presente trabalho, o qual se encontra estruturado em oito capítulos, iniciando-se com uma breve descrição do estado de arte (Introdução Geral - Capítulo 1), sendo de seguida fornecida informação detalhada acerca de cada uma das actividades de investigação desenvolvidas (Capítulos 2 a 7), terminando com um capítulo dedicado à síntese dos resultados chave, conclusões finais e perspectivas de futuros trabalhos (Capítulo 8).

Para a realização dos estudos apresentados contámos com a colaboração da Sortegel, Produtos Congelados, S.A. (Sortes-Bragança), principal empresa no sector da transformação industrial de castanha em Portugal. Assim, de acordo com a linha de processamento adoptada e implantada nesta empresa, seleccionaram-se as seguintes etapas para a recolha das amostras destinadas às diversas determinações analíticas: A - após recepção das castanhas pela empresa e no momento da calibração (etapa controlo); B - após três meses de conservação a ±0°C e humidade relativa de 90%; C - após descasque industrial por queima a uma temperatura de 800-1000 ºC durante 1 a 2 segundos; D - após congelação num túnel com um fluxo de CO_2 a -65 °C durante 15 a 20 minutos.

Em cada campanha e para cada uma das cultivares escolhidas para estudo, constituiu-se um lote com aproximadamente 400kg de castanhas com a mesma origem/proveniência (souto de um médio a grande produtor). De cada lote colheram-se aleatoriamente três amostras (repetições) em cada uma das etapas atrás referidas da linha de processamento. Cada amostra colhida foi subdividida em várias sub-amostras, consoante a natureza dos diversos tipos de análises efectuadas. Avaliou-se o efeito da cultivar, da etapa de processamento e da interacção cultivar x etapa de processamento no conteúdo de importantes nutrientes e não-nutrientes, e calculou-se a

contribuição de cada um destes factores para a variação total observada nos diversos constituintes determinados.

Num estudo preliminar (Capítulo 2), relativo à campanha de 2005, procedeu-se a uma análise comparativa da composição química das amostras colhidas à recepção/calibração (etapa A) das três mais importantes cultivares portuguesas de castanha - Judia, Longal e Martaínha. Os valores obtidos revelaram diferenças significativas entre as três cultivares para a maioria dos parâmetros estudados. No conjunto das análises efectuadas, estas cultivares apresentaram elevados teores de amido (57,5-64,9g $100g^{-1}$ MS) e apreciáveis níveis de constituintes da parede celular (NDF - 12,6 a 16,9g $100g^{-1}$ MS), não sendo de menosprezar as concentrações de proteína bruta (3,9-5,2g $100g^{-1}$ MS), gordura bruta (1,6-2,6g $100g^{-1}$ MS) e cinzas totais (1,8-2,3g $100g^{-1}$ MS). Observou-se um efeito significativo da cultivar no teor de aminoácidos livres, incluindo vários aminoácidos essenciais. Estas cultivares apresentaram um conteúdo significativo de polifenóis, com predomínio dos ácidos gálico e elágico (3,5-9,1 e 2,7-10,5mg g^{-1} peso fresco, respectivamente) entre os taninos hidrolisáveis e condensados, os quais são conhecidos pelos seus efeitos positivos na saúde.

Nesse mesmo estudo, procedeu-se também à avaliação do efeito das etapas de processamento acima descritas na composição química das cultivares Longal e Judia. A análise de variância com base num factor de variação demonstrou existirem, para a maioria dos constituintes analisados, diferenças significativas entre estas duas cultivares em cada uma das etapas de processamento amostradas. Por sua vez, a análise de variância com base em dois factores de variação revelou que a etapa de processamento e a interacção cultivar x etapa de processamento foram os factores que mais contribuíram para a variação observada.

Os estudos que se seguiram reportam-se às campanhas de 2006 e 2007, recaindo o primeiro destes anos sobre as três cultivares portuguesas já consideradas na campanha de 2005, isto é Judia, Longal e Martaínha, e duas cultivares estrangeiras conhecidas pelas sua boa aptidão para a transformação, uma de origem espanhola, mais propriamente da região da Galiza e designada por "Puga do Bolo", e outra originária da região de Viterbo (Itália) e conhecida por "Marron Vero" ou "Viterbes". Na campanha de 2007, do conjunto de cultivares a estudar foi excluída a "espanhola" e introduzida, em seu lugar, a variedade portuguesa Lada. Esta opção baseou-se, por um lado, no fraco poder de conservação registado na cultivar "espanhola" e, por outro lado, no facto de existirem indicações de que a cultivar Lada parece possuir características tecnológicas favoráveis ao processo de transformação, o que naturalmente interessava averiguar. Por sua vez, apesar das características apresentadas justificarem que a "italiana" continuasse a integrar o conjunto de cultivares a analisar no âmbito dos estudos previstos, não foi possível à

Sortegel garantir o seu fornecimento. Assim, nesta campanha os estudos incidiram nas mesmas três principais cultivares portuguesas que já tinham feito parte do lote de cultivares analisadas nas duas anteriores campanhas, às quais se juntou a Lada.

Os resultados das análises efectuadas nas amostras de castanha referentes às campanhas de 2006 e 2007 encontram-se apresentados e discutidos nos artigos correspondentes aos Capítulos 3 (matéria seca, cinzas totais, amido, gordura e energia bruta), 4 (proteína bruta, aminoácidos livres e compostos fenólicos) e 5 (minerais, açúcares livres, carotenóides e vitaminas C e E).

Para a totalidade de cultivares e etapas de processamento analisadas, registaram-se elevados conteúdos de amido (48,7-54,9g $100g^{-1}$ MS), responsáveis pelos altos níveis de energia bruta (410,1-427,8kcal $100g^{-1}$ MS), e teores relativamente baixos de proteína bruta (4,1-5,4g $100g^{-1}$ MS) e principalmente de gordura bruta (1,9-4,4g $100g^{-1}$ MS). Em ambos os anos de colheita, a interacção cultivar x etapa de processamento foi o factor que mais contribuiu para a variação observada nos teores de amido, enquanto que a variação ao nível dos teores de matéria seca e de fibra ficou a dever-se mais ao efeito da cultivar e da etapa de processamento, respectivamente.

No conjunto dos aminoácidos essenciais a Thr apresentou os valores mais elevados (3,6-10,0mg $100g^{-1}$ PF), enquanto que a Asn (14,9-36,4mg $100g^{-1}$ PF) foi o mais representativo entre os aminoácidos não essenciais presentes na totalidade das amostras.

Entre os factores testados em cada ano de estudo, a etapa de processamento foi o que mais contribuiu para a variação observada no conteúdo de compostos fenólicos totais (6,6-18,3mg g^{-1} PF). Na campanha de 2007, este factor, juntamente com a interacção cultivar x etapa de processamento, foram os principais responsáveis pela variação ocorrida nos teores de ácido gálico, enquanto que a variação nos teores de ácido elágico dependeu em maior proporção da cultivar.

Relativamente à composição em sais minerais, o fósforo (99,8-156,4mg $100g^{-1}$ MS) e principalmente o potássio (629,2-811,1mg $100g^{-1}$ MS) constituem os elementos predominantes em todas as amostras analisadas, e outros minerais com funções metabólicas importantes, tais como o cálcio, magnésio, ferro, zinco e manganês foram também encontrados.

Entre os açúcares livres, cuja variação no conteúdo total foi determinada em maior grau pela etapa de processamento, em ambos os anos de colheita, a sacarose assumiu uma posição de destaque (8,8-14,9g $100g^{-1}$ MS).

Foram também encontrados teores assinaláveis de luteína (0,1-0,6µg g^{-1} PF), ésteres de luteína (0,1-0,4µg g^{-1} PF) e vitamina C (4,2-7,2 e 3,1-6,8mg $100g^{-1}$ PF, para os ácidos ascórbico e desidroascórbico, respectivamente).

À semelhança do verificado anteriormente, estes estudos confirmam a existência de uma notória variação na composição química e valor nutritivo da castanha, em função quer da cultivar, quer do ano de colheita. Mas foi igualmente demonstrado, pela primeira vez, que essa variação é também determinada pelo processamento industrial inerente à obtenção de castanha congelada. Com efeito, sendo certo que este processamento permite o alargamento do período de armazenamento e comercialização da castanha, possibilitando o seu consumo mais regular e praticamente durante todo o ano, os estudos realizados permitiram concluir que a composição química e nutricional da castanha foi afectada, tanto negativa como positivamente.

Assim, em termos gerais e ao comparar os valores obtidos nas amostras em fresco (etapa A) e nas amostras congeladas (etapa D), observou-se um acréscimo significativo nos teores de NDF (12,7-19,9 e 16,3-18,2g $100g^{-1}$ MS), compostos fenólicos totais (6,6-12,0 e 10,6-16,9mg g^{-1} PF), Thr (4,9-6,8 e 3,6-7,9mg $100g^{-1}$ PF) e Ala (3,3-6,5 e 4,0-5,7mg $100g^{-1}$ PF) em 2006 e 2007, respectivamente, bem como de δ-tocoferol (0,3-0,4µg g^{-1} PF em 2006) e γ-tocoferol (25,2-27,3µg g^{-1} PF em 2007), enquanto que os teores de amido total (53,8-51,3 e 54,9-51,0g $100g^{-1}$ MS), Glu (14,7-9,2 e 13,9-10,9mg $100g^{-1}$ PF) e ácido ascórbico (6,4-5,4 e 7,2-5,1mg $100g^{-1}$ PF) em 2006 e 2007, respectivamente, e ainda de gordura bruta (2,9-2,5g $100g^{-1}$ MS em 2007), sofreram um decréscimo estatisticamente significativo.

Num outro estudo (Capítulo 6) procedeu-se à análise da composição química dos resíduos resultantes do processo de descasque, isto é, pericarpo (casca externa) e tegumento (casca interna), os quais representaram em média entre 8,9 e 13,5% e entre 6,3 e 10,1% do peso total dos frutos, respectivamente. Estes componentes são gerados em grandes quantidades e possuem um elevado potencial como fonte de valiosos co-produtos. Assim, avaliou-se a composição em polifenóis e taninos das amostras de pericarpo e tegumento das castanhas colhidas nas etapas A e B do processamento industrial e pertencentes às quatro cultivares nacionais (Judia, Longal, Martaínha e Lada) analisadas na campanha de 2007. Registaram-se assinaláveis teores de fenólicos totais e de baixo peso molecular (ácidos gálico e elágico), taninos condensados e elagitaninos, incluindo castalagina, vescalagina, acutissimina A e acutissimina B. Os valores mais elevados de fenólicos totais e taninos condensados foram encontrados ao nível do tegumento. Neste estudo, testaram-se também vários meios de extracção a duas diferentes temperaturas (20 e 70 ºC), verificando-se que a solução acetona:água (70:30) a 20 ºC foi a que permitiu uma maior extracção, não só de fenólicos totais (68,5-136,4mg g^{-1} PF)

nos dois tipos de amostras e para a totalidade das cultivares analisadas, como de taninos condensados (16,3-110,4mg g^{-1} PF) e compostos fenólicos de baixo peso molecular nas amostras (pericarpo e tegumento) da cultivar Longal. Ao nível do pericarpo desta cultivar, os valores de ácido elágico, tanino T1, acutissimina B e um derivado do ácido elágico variaram significativamente consoante os solventes utilizados na extracção. Este estudo revelou que, na cultivar Longal, os valores mais elevados, tanto ao nível do pericarpo como do tegumento, correspondem ao ácido gálico e à castalagina.

O último estudo (Capítulo 7) corresponde a um artigo de revisão bibliográfica que pretende retratar o estado actual de conhecimentos acerca da composição da castanha (semente ou parte edível) de origem europeia (*Castanea sativa* Mill.) em nutrientes e não-nutrientes (fitoquímicos) relacionados com a saúde, de que forma esta composição é afectada pelo processamento artesanal e industrial, capaz de originar diferentes formas de utilização deste fruto, e quais as consequências na qualidade dos alimentos dele derivados e seus eventuais efeitos na saúde dos consumidores.

Embora a investigação já realizada constitua um contributo assinalável para um melhor conhecimento dos benefícios para a saúde associados à ingestão de castanha, outros estudos são todavia necessários, no sentido quer de maximizar a produção, quer de melhorar a qualidade dos produtos obtidos a partir deste fruto (e.g. selecção e melhoramento genético; ajustamento e optimização do processamento industrial). Por outro lado, torna-se cada vez mais imprescindível: *i*) a avaliação da actividade enzimática, de forma a obter uma melhor compreensão das alterações que ocorrem durante as principais etapas do processamento e transformação industrial; *ii*) a realização de investigação com modelos celulares, capaz de avaliar os efeitos ao nível da digestão, absorção e metabolismo em humanos, de forma a comprovar os potenciais benefícios para a saúde e o efectivo valor nutricional deste fruto.

Palavras-Chave: *Castanea sativa* Mill.; castanha; processamento industrial; alterações na composição química; nutrientes; minerais; fitoquímicos; co-produtos.

ABSTRACT

Chestnut trees are cultivated throughout southern Europe (mainly Italy, Portugal, France and Spain), Turkey, North America, South America (Chile and Bolivia), Asia (China, Japan and Korea), Australia and New Zealand, where the fruits are widely consumed. These fruits contain starch, free sugars, fibre, proteins, lipids, vitamins, and minerals, as well as other bioactive non-nutrients such as polyphenolics. Chestnuts are recognized as a rich source of beneficial health compounds. However, there have been few studies on the changes that occur in the chemical composition of chestnut fruits during industrial processing, and that is the main purpose of this work, which is structured into eight chapters beginning with a description of the state of the art (General Introduction - Chapter 1) and then detailed information is presented about each of the research activities (Chapters 2 to 7) followed by a chapter dedicated to the key results obtained, the concluding remarks and future work perspectives (Chapter 8).

In order to perform the studies presented in the chapters 2 to 6 of this work, a collaboration was established with Sortegel, Produtos Congelados, S.A. (Sortes-Bragança), the main company in the chestnut fruit processing sector in Portugal. Thus, according to the processing line adopted by this company, the following stages were selected for the collection of the samples used for the various analytical determinations: A - after reception of the chestnut fruits at the company and at the moment of calibration (control stage); B - after 3 months storage at ±0 °C and relative humidity of 90%; C - after industrial flame peeling at 800-1000 ºC during 1 to 2 seconds; D - after freezing in a tunnel with a CO_2 flow at -65 °C during 15 to 20 minutes.

In each harvest year, and for each one of the selected cultivars in these studies, a lot of 400 kg of chestnut fruits with the same origin (orchard of a medium or large producer) was gathered. From each lot three samples (repetitions) were randomly collected of each stage of the referred processing stages. Each one of the samples collected was subdivided into several sub-samples, according to the nature of the different analyses performed. The effect of the cultivar, processing stage and interaction between these two factors on the content of important nutrients and non-nutrients was evaluated, also calculating the contribution of each factor to the total variation of the different determined constituents.

In the first study (Chapter 2), analyzing samples from the 2005 harvest, a comparative analysis was performed on the chemical composition of the samples collected at reception/calibration (stage A) of the three main Portuguese chestnut cultivars (Judia, Longal and Martaínha). The values obtained showed significant differences among the three cultivars for

most of the studied parameters. From the analyses performed the studied cultivars had high starch contents (57.5-64.9g $100g^{-1}$ DM) and relevant levels of cell wall constituents (NDF - 12.6-16.89g $100g^{-1}$ DM), but also not inconsiderable contents of crude protein (3.9-5.2g $100g^{-1}$ DM), crude fat (1.6-2.6g $100g^{-1}$ DM) and total ash (1.8-2.3g $100g^{-1}$ DM). The cultivar factor significantly influenced the variation observed for the free amino acid contents, including various essential amino acids. These cultivars had a significant content of polyphenolics with gallic and ellagic acids (3.5-9.1 and 2.7-10.5mg g^{-1} FW, respectively) being predominant among the hydrolysable and condensed tannins that are known for their positive effects on health.

In the same study, an evaluation was made on the effects of the processing stages on the chemical composition of the cultivars Longal and Judia. The one-way analysis of variance showed that the cultivar factor (Judia and Longal at the four processing steps) significantly affected almost all the constituents. On the other hand, the two-way analysis of variance shows that the processing step and the interaction cultivar x processing step were the factors that had more of a contribution to the total variation observed.

In the next three studies the effects of the selected processing stages on the chemical composition of the chestnut cultivars from the 2006 and 2007 harvest years, were analysed. In 2006, the cultivars analyzed were: three Portuguese cultivars (Judia, Longal e Martaínha) already present in the 2005 harvest year, and two foreign known for their good transformation characteristics, one of Spanish origin from the region of Galicia named "Puga do Bolo" and the other from the region of Viterbo (Italy) known as "Marron Vero" or "Viterbes". In the harvest year of 2007, of the cultivars selected for analysis the "Spanish" one was substituted by the Portuguese cultivar Lada. This option was based on the poor storage characteristics revealed by the "Spanish" cultivar, and on the other hand also related to the fact that Lada presented good technological characteristics in relation to the transformation process, which was of high interest for this study. Although the characteristics presented by the "Italian" cultivar justified the continuation in this study, Sortegel was unable to ensure the supply of this cultivar. Therefore, in this harvest year the selected cultivars for analysis were the three main Portuguese analyzed in the previous two harvest years with the addition of the cultivar Lada.

The results of the samples analyzed from the harvest years 2006 and 2007 are presented in Chapters 3 (dry matter, total ash, starch, fat and crude energy), 4 (crude protein, free amino acids and polyphenolics) and 5 (mineral, free sugars, carotenoids and vitamins C and E).

Considering all the cultivars and processing stages analyzed, the samples had high amounts of starch (48.7-54.9g $100g^{-1}$ DM) responsible for the significant contents of crude energy (410.1-427.8kcal $100g^{-1}$ DM) and low contents of crude protein (4.1-5.4g $100g^{-1}$ MS) and

especially fat (1.9-4.4g $100g^{-1}$ DM). For both harvest years, the interaction cultivar x processing stage had the greatest influence on the starch contents while the cultivar and processing stage factors had a much greater influence on the dry matter and fibre contents, respectively.

Of the group of the essential amino acids, Thr had the highest values (3.6-10.0mg $100g^{-1}$ FW), while Asn (14.9-36.4mg $100g^{-1}$ FW) had the highest value of the non-essential amino acids present in the samples.

The total phenolics (6.6-18.3mg g^{-1} FW) contents were more significantly affected by the processing stage factor. In the 2007 harvest year the gallic acid contents were more affected by the processing stage and by the interaction cultivar x processing stage, while the cultivar factor had more of an effect on the ellagic acid contents.

The minerals phosphorous (99.8-156.4mg $100g^{-1}$ DM) and mainly potassium (629.2-811.1mg $100g^{-1}$ DM) were predominant in all analysed samples and other health-related minerals such as calcium, magnesium, iron, zinc and manganese were also present.

The free sugar contents, which were more influenced by the processing stage in both harvests, showed that sucrose (8.8-14.9g $100g^{-1}$ DM) was predominant.

Significant levels of lutein (0.1-0.6µg g^{-1} FW), lutein esters (0.1-0.4µg g^{-1} FW), γ-tocopherol (4.1-27.3µg g^{-1} FW), vitamin C (4.2-7.2 and 3.1-6.8mg $100g^{-1}$FW for ascorbic and dehydroascorbic acids, respectively) were also found.

In the current studies the fruit composition was clearly influenced either by the cultivar or harvest year, as shown in previous studies. This work also revealed, for the first time, that this composition is affected by the industrial processing stages related to the chestnut frozen market line. It is a fact that this processing allows the extension of the shelf life (storage time) and commercialization of chestnut fruits, and therefore the consumption during all year. The results also show that the industrial processing of chestnut fruits leads to both quantitative "positive" and "negative" effects in the chemical and nutritional composition.

Thereby, comparing the values obtained from the fresh (stage A) and frozen (stage D) samples, there were significant increases in the contents of NDF (12.7-19.9 and 16.3-18.2g $100g^{-1}$ DM), total phenolics (6.6-12.0 and 10.6-16.9mg g^{-1} FW), Thr (4.9-6.8 and 3.6-7.9mg $100g^{-1}$ FW) and Ala (3.3-6.5 and 4.0-5.7mg $100g^{-1}$ FW) in 2006 and 2007, respectively, as well of δ-tocopherol (0.3-0.4µg g^{-1} FW in 2006) and γ-tocopherol (25.2-27.3µg g^{-1} FW in 2007), while the amounts of total starch (53.8-51.3 and 54.9-51.0g $100g^{-1}$ DM), Glu (14.7-9.2 and 13.9-10.9mg $100g^{-1}$ FW) and ascorbic acid (6.4-5.4 and 7.2-5.1mg $100g^{-1}$ FW) in 2006 e 2007, respectively, and also of fat (2.9-2.5g $100g^{-1}$ DM in 2007), showed a statistically significant decrease.

In a parallel study (Chapter 6) various analyses of the chestnut fruit processing residues resulting from the industrial peeling were done, which are the pericarp (outer shell) and integument (inner shell), representing in average between 8.9 to 13.5% and 6.3 to 10.1% of the total fruit weight, respectively. These waste materials are created in large amounts and have a great potential to become sources of valuable co-products. Therefore the pericarp and integument of four Portuguese chestnut cultivars (Judia, Longal, Martaínha and Lada) of the 2007 harvest year (stages A and B from the industrial processing) were analyzed for total phenolics, low molecular weight phenolics (gallic and ellagic acid), condensed tannins and ellagitannins including castalagin, vescalagin, acutissimin A and acutissimin B. The highest levels of total phenolics and condensed tannins were found in the integument tissues. Different extraction solvents were tested at two temperatures (20 ºC and 70 ºC). The extraction solvent 70:30 acetone:water at 20 ºC was the most efficient for the extraction of total phenolics (68.5-136.4mg g^{-1} FW), and in Longal of total condensed tannins (16.3-110.4mg g^{-1} FW) and low molecular weight phenolics. In the cultivar Longal the values obtained of ellagic acid, tannin T1, acutissimin B and an unidentified ellagic acid derivative in the pericarp were significantly different depending on the extraction solvent used. In Longal the shells (pericarp and integument) revealed higher amounts of gallic acid and castalagin.

The final study (Chapter 7) is a review of the current knowledge of the composition of health-related nutrients and non-nutrients (phytochemicals) in European chestnut (*Castanea sativa* Mill.) fruits (seed or kernel), and an evaluation of how this composition is affected by home and industrial processing, which provides different forms of utilization of this fruit and the subsequent consequences on the quality of the derived food products in relation to health effects.

Although the original research performed for this thesis constitutes an important contribution for a better understanding of the benefits provided from chestnut consumption for human and animal health, further studies need to be performed to optimize the production and quality of chestnut-derived materials (e.g. genetic selection and industrial processing). On the other hand it is crucial to: *i*) evaluate enzymatic activities in order to obtain a better knowledge of the changes that occur during the main processing stages; *ii*) perform research using human cell models and human intervention studies to prove the effective nutritional and health value of the chestnut fruits.

Key Words: *Castanea sativa* Mill.; chestnut fruits; industrial processing; compositional changes; nutrients; minerals; phytochemicals; co-products.

OBJECTIVES

The main objectives of this work were *i*) to evaluate the quality of the major cultivars of Portuguese chestnut fruits, and compare with the fruits of two other European cultivars (one Spanish and one Italian), in relation to their nutrient and non-nutrient (phytochemical) composition and *ii*) to comprehensively study the effects of industrial processing stages on the chemical and nutritional composition within these cultivars in order to identify those cultivars with optimal processing qualities.

To achieve this goal specific studies, with defined objectives, were set as follows:

1. Determine the composition of nutrients (total starch, crude fat, crude protein, total fibre, and free amino acids) and phytochemicals (total and specific phenolics) in the fresh fruits of three main Portuguese chestnut cultivars (Judia, Longal and Martaínha) from the 2005 harvest. Determine the effects at each of the different stages of industrial processing on the nutrients and phytochemicals in the fruits of Judia and Longal in order to identify possible improvements for the processing methods and also for the selection of cultivars with optimal composition and processing characteristics and thus optimal health effects (**Chapter 2**).
2. Determine the effects of the industrial processing stages (storage, peeling and freezing) on the contents of total starch, crude fat, crude energy and total fibre in the fruits of four Portuguese cultivars (Judia, Longal, Martaínha and Lada) and two selected European cultivars (one Spanish - "Puga do Bolo" from Galiza region; one Italian - "Viterbes"/"Marron Vero" from Viterbo region) from two harvests (2006 and 2007). Therefore identifying the most suitable cultivars for processing that maintain high levels of nutrients and other biologically-active compounds for optimal composition and quality (**Chapter 3**).
3. Evaluate in detail, the changes in crude protein, free amino acids and phenolic phytochemicals during the industrial processing stages of the fruits of four previously defined Portuguese and two European cultivars from 2006 and 2007 harvest years (**Chapter 4**).
4. Evaluate in detail, the changes in minerals, free sugars, antioxidant vitamins and pigments during the industrial processing stages of the fruits of four previously defined Portuguese and two European cultivars from 2006 and 2007 harvest years (**Chapter 5**).

5. Determine the levels and profiles of tocopherols, pigments (carotenoids and chlorophylls) and specific phenolics (simple phenolics, ellagitannins and condensed tannins) in the pericarps and integuments of four Portuguese chestnut cultivars (pre- and post-storage from the 2007 harvest). Evaluate the extraction efficiencies for the phenolics using different low cost solvents, and thus determine the potential of these waste materials as sources of useful added-value phytochemical co-products (**Chapter 6**).
6. To complete a detailed review of the current knowledge on the composition of health-related nutrients and non-nutrients (phytochemicals) in chestnut fruits and how their composition can be affected by home and industrial processing and thus can affect the quality and health effects of the foods derived from chestnut (**Chapter 7**).

PUBLICATIONS

This thesis consists of five scientific papers published in peer reviewed international journals, and one paper that is accepted/in press, as a result of the original research performed and are presented below:

De Vasconcelos, M.C.B.M., Bennett, R.N., Rosa, E.A.S., Ferreira-Cardoso, J.V., 2007. Primary and secondary metabolite composition of kernels from three cultivars of Portuguese chestnut (*Castanea sativa* Mill.) at different stages of industrial transformation. *Journal of Agricultural Food and Chemistry*, **55:**3508-3516.

De Vasconcelos, M.C.B.M., Bennett, R.N., Rosa, E.A.S., Ferreira-Cardoso, J.V., 2009a. Industrial processing effects on chestnut fruits (*Castanea sativa* Mill.) 1. Starch, fat, energy and fibre. *International Journal of Food Science and Technology*, **44**:2606-2612**.**

De Vasconcelos, M.C.B.M., Bennett, R.N., Rosa, E.A.S., Ferreira-Cardoso, J.V., 2009b. Industrial processing effects on chestnut fruits (*Castanea sativa* Mill.) 2. Crude protein, free amino acids and phenolic phytochemicals. *International Journal of Food Science and Technology*, **44**:2613-2619.

De Vasconcelos, M.C.B.M., Nunes, F., García-Viguera, C., Bennett, R.N., Rosa, E.A.S., Ferreira-Cardoso, J.V., 2010. Industrial processing effects on chestnut fruits (*Castanea sativa* Mill.) 3. Minerals, free sugars, carotenoids and antioxidant vitamins in chestnut fruits (*Castanea sativa* Mill.). *International Journal of Food Science and Technology*, **45**: 496-505**.**

De Vasconcelos, M.C.B.M., Bennett, R.N., Quideau, S., Jacquet, R., Rosa, E.A.S. & Ferreira-Cardoso, J. V., 2010. Evaluating the potential of chestnut (*Castanea sativa* Mill.) fruit pericarp and integument as a source of tocopherols, pigments and polyphenols. *Industrial Crops and Products*, **31**:301-311.

De Vasconcelos, M.C.B.M., Bennett, R.N., Rosa, E.A.S. & Ferreira-Cardoso, J. V., 2010. Composition of European chestnut (*Castanea sativa* Mill.) and association with health effects: fresh and processed products. *Journal of the Science of Food and Agriculture* **(Accepted/In Press).**

LIST OF ABBREVIATIONS

ADF……... Acid Detergent Fibre
ADL……... Acid Detergent Lignin
ALA……... Alpha-Linolenic Acid
Asc…… Ascorbic Acid
DHAsc……... Dehydroascorbic Acid
CE…….. Crude Energy
DM…… Dry Matter
DHA……... Docosahexaenoic Acid
EA…….. Ellagic Acid
GA …… Gallic Acid
EPA…….... Eicosapentaenoic Acid
EXP…… Exportation
FW…… Fresh Weight
GABA…… Gamma-Amino Butyric Acid
HLA……... Human Leucocyte Antigen
IMP…… Importation
INE…… Instituto Nacional de Estatística
LDL……... Low Density Lipoprotein
MDHAsc…… Monodehydroascorbic Acid
MS…….. Matéria Seca
MUFA…… Monounsaturated Fatty Acids
NDF……... Neutral Detergent Fibre
NDS……... Neutral Detergent Solubles
PF……... Peso Fresco
PUFA……. Polyunsaturated Fatty Acids
RDI…… Recommended Daily Intake
RH…….. Relative Humidity
SCFA……. Short Chain Fatty Acids
SFA…… Saturated Fatty Acids
USDA…… United States Department of Agriculture

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

The chestnut tree (*Castanea sativa* Miller) is one of the most important agro-forestry species in the Northern and Central regions of Portugal and in many other European countries. Especially in Trás-os-Montes the optimum ecological and climatic conditions ensure the survival and development of this nut tree. Chestnut species are important sources of raw materials for various industrial applications (wood for furniture and wine barrels, tannins for leather processing), and the fruits are an important food in Europe (mainly Italy, Portugal, France and Spain), Turkey, North and South America and Asia (China, Japan and Korea) (Tables 1.1 and 1.2).

Table 1.1 - World production of chestnut fruits (10 major producers) (Source: FAO - FAOSTAT Statistics Division, 2009 and 2010).

Year	Chestnut Production (t)									
	China	**North Korea**	**Turkey**	**Italy**	**Japan**	**Bolivia**	**Portugal**	**Greece**	**France**	**Spain**
1995	300000	93655	77000	71971	34400	31229	23238	12053	11016	10075
1996	285000	108346	65000	68653	30100	39565	25272	12536	10798	15000
1997	375000	129673	61000	72782	32900	39872	26357	13548	9592	10000
1998	450000	109956	55000	78425	26200	30864	29314	12820	11411	11852
1999	534631	95768	53000	52158	30000	33603	30969	15733	12563	10580
2000	598185	92844	50000	50000	26700	34400	33317	15303	13224	9230
2001	599077	94130	47000	50000	29000	40000	26118	14935	13032	9510
2002	701684	72405	47000	50000	30100	46000	31385	15200	11223	9362
2003	797168	60017	48000	50000	25100	50000	33267	16800	10118	16821
2004	922735	71795	49000	50000	24000	52758	31051	18712	12431	9510
2005	1031857	76447	50000	52000	21800	57057	22327	20946	8144	8629
2006	850000	82450	53814	53000	23100	55000	30900	17442	9670	10140
2007	925000	77524	55100	55000	22100	55000	22000	10200	8284	15000
2008	925000	80000	55395	55000	22100	55000	22000	9800	6258	15000

Table 1.2 - World production of chestnut fruits (minor producers) (Source: FAO - FAOSTAT Statistics Division, 2009)*.

Year	Chestnut Production (t)										
	South Korea	Russian Fed	Peru	Serbia Mont.	Hungary	Romania	Azerbaijan	Rep of Macedonia	Switzerland	Albania	Bulgaria
1995	7500	4600	2215	1400	1100	1000	500	300	200	-	-
1996	7500	4500	2202	1400	1040	1000	400	700	-	700	350
1997	7500	4200	2070	1400	991	1000	300	600	-	700	300
1998	8400	4500	750	1400	973	-	300	500	200	500	200
1999	8400	4200	1954	1100	1029	-	400	200	200	400	200
2000	8400	3800	1990	900	1015	-	400	250	200	400	400
2001	8700	4000	525	700	991	-	500	200	200	400	300
2002	8700	4000	670	400	580	-	500	300	200	380	300
2003	9000	4000	759	900	469	-	937	400	200	480	200
2004	9000	2000	775	900	444	-	949	300	200	380	100
2005	9000	3500	600	900	385	-	1937	300	200	380	100
2006	8000	2100	800	-	426	-	1765	300	252	500	100
2007	9000	2200	800	-	338	-	1887	320	254	500	100

* Data not available for 2008.

The decrease of chestnut production in Portugal was specially marked in the middle of the 19th century with the appearance of other food sources such as potatoes, maize and rye. Chestnut tree productivity was also affected by economic factors (expensive wood) and by the appearance of the "ink disease" associated with the soil-borne pathogens *Phytophthora cinnamomi* Rands and *Phytophthora cambivora* (Petri) Buis. Later in the beginning of the 20th century the appearance of the chestnut canker (caused by the fungus *Cryphonectria parasitica* (Murr.) Barr.) caused further significant decreases of this tree´s productivity (Adua, 1999).

According to Ferreira-Cardoso (2002) the development of adequate industrial units, will contribute to a better valorisation of this nut production. This evolution will certainly allow a significant increase in chestnut fruit exportations, either fresh or processed, as long this production is based on the utilisation of the best cultivars leading to an improvement of the actual production quality. The same author stated that the high economic value of chestnut trees is not only due to the fruit and wood productivity, but is also associated with other activities e.g. rural tourism and folk activities, which also play an important role in the management of synergistic resources as sources of food for several hunting species.

The main factors that limit the successful production and industrial processing of this nut are: (1) difficulties in the control of the ink and blight diseases in chestnut trees; (2) insufficient

industrial utilization of chestnut fruits and (3) the low industrial activity contributing to the absence of processed products, affecting the level of exportation from Portugal, while in other countries the consumption of products derived from this nut increases significantly (Ferreira-Cardoso, 2002). Chestnut fruit has been the subject of intensive research in the last decade, mainly because of the potential human health benefits that the intake of this nut might provide, assuming special importance as part of a balanced diet rich in antioxidant compounds, known for having positive health effects.

1.2 Biology of the Genus *Castanea*

The genus *Castanea* belongs to the sub-family Castaneoideae and family Fagaceae that includes other ecologically and economically important tree species, such as *Aesculus hippocastanum* (horsechestnut), *Betula pendula* (birch), *Fagus sylvatica* (beech) and *Quercus* species (oaks) (Manos *et al*., 2001). According to Gomes-Laranjo and Crespí (2007) the European chestnut tree was included in the genus *Castanea* in 1768 by the botanist of Scottish descent Phillip Miller and then onwards designated as *Castanea sativa* Mill. The chestnut tree is geographically distributed in three main areas: Asia with *Castanea crenata* Sieb. and Zucc. (Japan) and *Castanea mollissima* Bl. (China and Korea), North America with *Castanea dentata* Borkh. and Europe with *Castanea sativa* Mill. (Bounous, 2005). In Portugal, as in the rest of Europe, *Castanea sativa* Mill. is the most cultivated species.

1.2.1 Origin and Ecology

The hybrid species of chestnut appeared as a result of genetic crossing of *Castanea sativa* Mill. with other species, specifically *Castanea crenata* Sieb. & Zucc. from Japan and *Castanea mollissima* Blume from China. These hybrids offer benefits as rootstocks and provide resistant genes to the ink disease in the chestnut tree (Martins and Abreu, 2007).

The European chestnut (*Castanea sativa* Mill.) probably had its origins in the Eastern Mediterranean region over 90 million years ago in the middle Cretaceous period, later spreading throughout Europe during the Cenozoic period (Adua, 1999; Ferreira-Cardoso, 2002). Later, between 900-700 BC, deliberate chestnut tree cultivation occurred in the Asian regions of the Caspian and Black sea. After this time chestnut cultivation quickly spread to Greece and later the Romans discovered the benefits of this nut tree (Adua, 1999). However, Van Den Brink and Janssen (1985) showed that fossilized chestnut pollen found in “Serra da Estrela” was at least 8000 years old (Palaeolithic period), and therefore these findings prove that the appearance of

chestnut trees in Portugal may have occurred much earlier than the Roman occupation of the Iberian Peninsula.

The chestnut tree is a deciduous species with hypogeal germination and when grown under optimal soil and climatic conditions this nut tree has a rapid growth rate up to 70 to 80 years old, with an average height of 10 to 20 meters (Pinto *et al*., 2007). The same authors described the leaves as simple with sub-coriaceous consistency and disposed alternately. These trees possess a strong and robust root system with little development in depth (50cm), being able to reach long distances horizontally (Pinto *et al*., 2007). They have very low tolerance to limestone, to deficient drainage and to compacted soils, needing low acidity, and deep soils rich in organic matter and minerals i.e. potassium, sodium and magnesium (Portela *et al*., 2007). According to Valdiviesso *et al*. (1993) chestnut is a monoecious species and has two different types of inflorescences: the unisexual staminate catkins in the lower parts of the shoot and the bisexual catkins in the terminal end of the shoots (Figure 1.1). The staminate flowers are disposed in a spiral along the axis of the catkin in clusters (three to seven), while the pistillate inflorescences are arranged individually or in clusters (two or three) at the base of the bisexual catkins. The same authors state that the flowers usually present seven to nine styles. The flowering is dependant on climatic conditions during the spring season, occurring usually between May and middle July, with the masculine inflorescences appearing first. After flowering, cross-pollination occurs by insects and wind. The chestnut fruit requires a long warm period of 75 to 120 days to develop (Pinto *et al*., 2007). This happens inside the burr which opens when the fruit is fully mature. There is very little previous information about chestnut production yields, which are affected by factors such as cultivar, region and edaphoclimatic conditions. Ferreira-Cardoso and Pimentel-Pereira (2007) reported values of 30 to 40 kg/tree, while Lüdders (2004) reported higher production yields ranging between 100 to 150 kg/tree, with mature trees producing up to 300 kg.

The burr usually possesses 1 to 3 fruits (Figure 1.1), but more rarely 7 to 8 fruits can also appear. The fruit is formed from the fertilized ovum containing only one fertile seed (monospermic) or if each feminine flower has several ova then polyspermic fruits can develop (Bergougnoux *et al.,* 1978). According to the same authors in the first case the fruit is designated "marron", while in the second case it is named "chestnut". The shell of chestnut fruits consists of the pericarp (hard external shell) and the integument (soft internal shell or skin) (Figure 1.2).

Figure 1.1 - Feminine and masculine inflorescences (left) and mature burr with fruits (right).

Figure 1.2 - Chestnut fruit half peeled and shell - pericarp and integument (left) and peeled fruit with deep indentations (right).

1.2.2 Chestnut Species and Cultivars

Chestnut is geographically distributed in three major areas: Europe, Asia (Turkey, Japan, China and Korea), and North America (Figure 1.3).

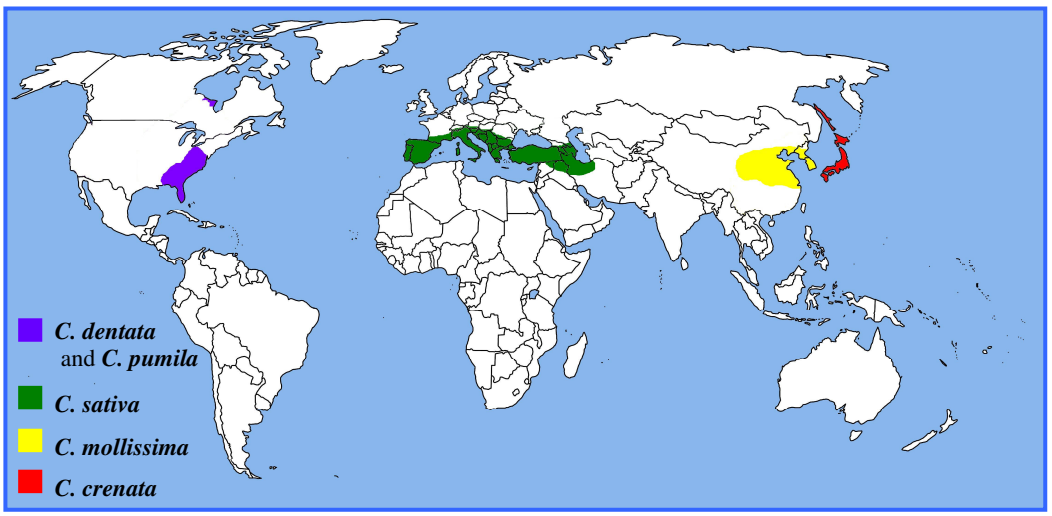

Figure 1.3 - World geographic distribution of chestnut species (Source: Lage, 2005).

The European chestnut tree (*Castanea sativa* Mill.) is spread predominantly over the Mediterranean countries and within major forest areas (Figure 1.4). The domesticated populations of this tree are formed into orchards (Lüdders, 2004).

In Portugal the chestnut trees are more intensely distributed in the regions of Trás-os-Montes and Beiras (Figure 1.5), but they are also found in some Northeastern areas of Alentejo - Marvão (mountain of S. Mamede), in the North of Algarve (mountain of Monchique) and in Minho Interior (mountain of Gerês), and also in several places of the Northern regions of Tejo. However, chestnut trees can also be found in more isolated regions such as the islands of Madeira ("Curral das Freiras" and "Serra de Água") and Azores (Ferreira-Cardoso, 2002).

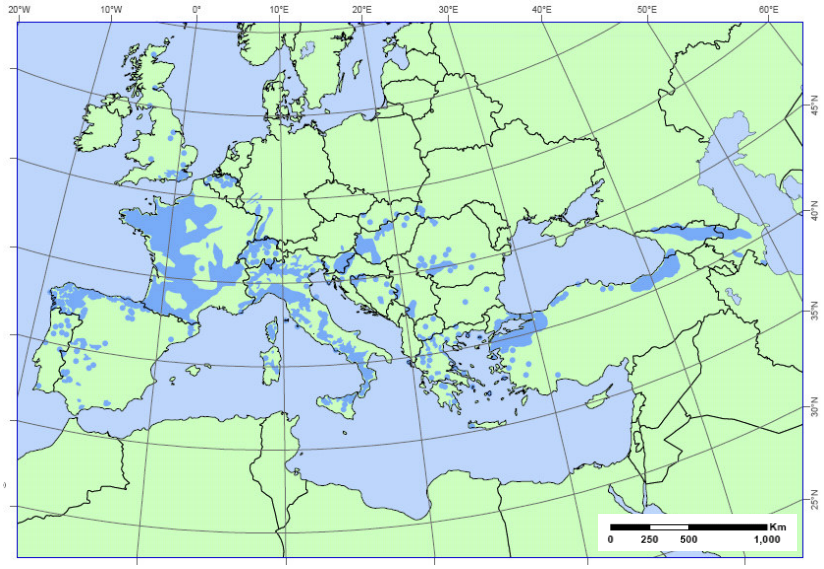

Figure 1.4

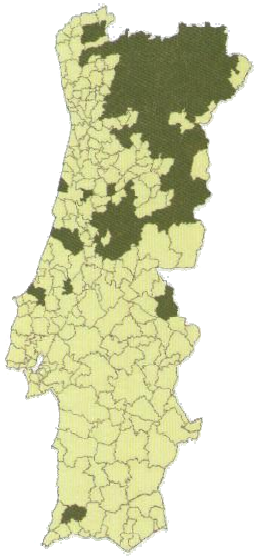

Figure 1.5

Figure 1.4 - European distribution of *Castanea sativa* Mill. (EUFORGEN, 2009).

Figure 1.5 - Chestnut (*Castanea sativa* Mill.) producing areas in Portugal (highlighted in green) (Lage, 2005).

1.2.2.1 General Characterization

Initially the naming of chestnut cultivars was based on phenotype alone and it is only more recently that genotype analyses have shown the specific relationships between regional and national cultivars. This has led to a certain degree of confusion with chestnut cultivars. There are several cultivars of chestnut in Portugal but sometimes different designations have been used for

the same cultivar in geographically close areas, or different cultivars from different/distant places have been given the same name. Further complexities have arisen when some cultivars, with apparently different origins and local designations, are derived from the same cultivar. This confusion occurs when they diverge morphologically due to environmental factors. A previous study with Portuguese chestnut cultivars detected the genetic variability of these cultivars by evaluating iso-enzyme polymorphisms (Pimentel-Pereira *et al*., 1999). This study was based on 7 different types of iso-enzyme profiles and it allowed the discrimination of 32 classes. To each class corresponded one or more distinct cultivars that were genetically very similar (synonymy), but in other cases genetically distinct cultivars had the same name (homonymy) (Pimentel-Pereira *et al*., 1999). A previous work of detection and inventory of the different Portuguese cultivars of chestnut and their geographic distribution is detailed in Table 1.3 (Ferreira-Cardoso and Pimentel-Pereira, 2007).

Table 1.3 - Geographic distribution of the different Portuguese cultivars (Ferreira-Cardoso and Pimentel-Pereira, 2007).

Districts	County	Cultivars
Braga	Terras de Bouro	Amarelal; Misericórdia; De Quarta
Bragança	Alfândega da Fé	Enxerta
	Bragança	Enxerta; Judia
	Carrazeda de Ansiães	Longal; Enxerta
	Macedo de Cavaleiros	Enxerta
	Mogadouro	Enxerta; Negra
	Vinhais	Judia (Carrazeda); Aveleira; Lamela; Trigueira; Boaventura; Longal
Guarda	Trancoso	Martaínha; Longal
Portalegre	Marvão	Bária; Colarinha; Enxerta (Longal)
Vila Real	Boticas	Pelada; Galizã; Longal
	Chaves	Vermelha; Longa
	Montalegre	Redonda; Nacional; Enxerta; Longal
	Murça	Cota; Longal
	Valpaços	Judia; Lada; Negral; Sousão; Lamela; Rebolão; Cota; Soutunha; Longal
	Vila Pouca	Cota; Longal; Bairral
	Vila Real (Campeã)	Benfeita; Bebim; Moreira; Longal
Viseu	Armamar	Riscal; Longal; Serrano
	Lamego	Cancela; Longal; Rabodiça
	Meda	Verdeal; Longal
	Moimenta da Beira	Negra; Demanda; Carreiró; Longal
	Penedono	Passã; Martaínha; Longal
	Sernancelhe	Martaínha; Longal

Thus it is possible to highlight the cultivars that are more known and commercially profitable (Ferreira-Cardoso and Pimentel-Pereira, 2007):

1. Judia, Longal, Lamela, Aveleira and Boaventura in the district of Bragança;
2. Judia, Longal, Lada, Negral, Cota and Benfeita in the district of Vila Real;
3. Martaínha and Longal in the districts of Viseu and Guarda;
4. Bária, Colarinha and Enxerta in the district of Portalegre.

1.3 Production and Economic Importance

1.3.1 National and International Production of Chestnut

For a long time in Portugal the chestnut fruit was the basis of the rural population's diet and was one of the major dietary starch sources, functioning as an important food source. However, chestnuts have gradually been replaced by potatoes in the current diet. Even the lower quality chestnut fruits were used, primarily in animal feeds. Chestnut trees were also used as sources of tannin-rich tissues (tannin contents of the wood confers high resistance to insect and fungal damage), with high levels of both procyanidins (condensed tannins) and ellagitannins (hydrolyzable tannins). This property has been exploited by using the wood for building materials, furniture and wine barrels (organoleptic properties in matured wines and brandies); although in current wine production oak barrels are now more commonly used (Canas *et al*., 1999). Tannins from the bark and wood of chestnut trees are also the prime sources for leather processing (Korel and Balaban, 2009).

According to the data published by the Statistics Division of FAO the production of chestnut fruit in Portugal increased steadily from 1995 to 2000, but from 2001 to 2008 there was considerable annual variation in production, and in 2007 and 2008 the production values were the lowest since 1995 (Figure 1.6). The chestnut fruit exportation market is one of the major ones in Portugal, when compared with other nuts, maintaining a positive balance in the commercial sector. The major importers/buyers of Portuguese chestnuts in 2008 were Spain (2 088t), France (1 407t), Brazil (1 186t) and Italy (419t), gathering in total 92.1% of the sales, while the remaining 7.9% were exported to Switzerland, United States of America, Canada, United Kingdom, Germany, Angola, Luxembourg, Cape Verde, South Africa and Belgium (GPP, 2010).

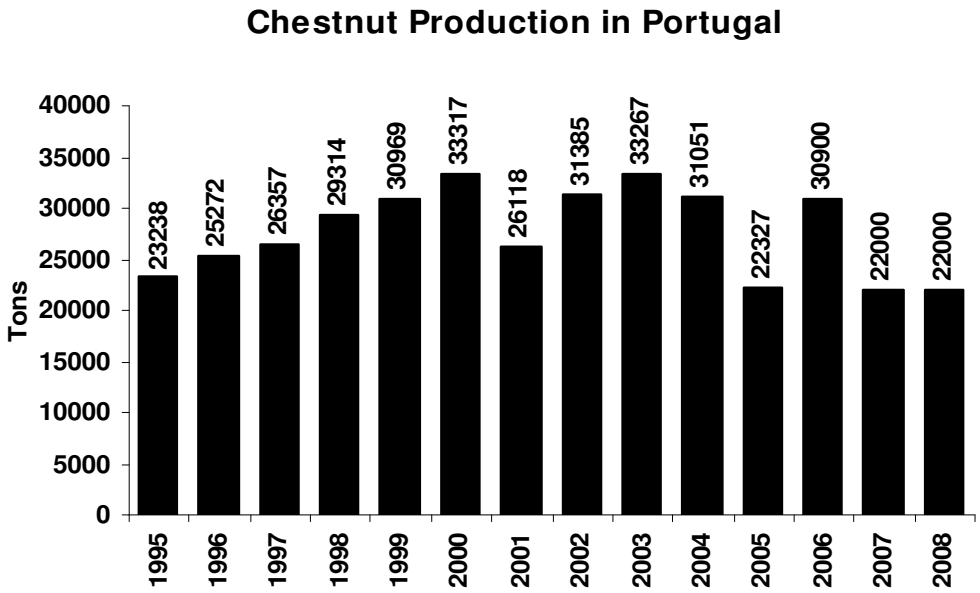

Figure 1.6 - Chestnut fruit production in Portugal (1995 - 2008) (Source: FAO - FAOSTAT Statistics division, 2009 and 2010).

The level of exportations from Portugal, between the year 2005 and 2007, reached the highest level in 2006 with 8 638t, while the level of importations to Portugal were lower in the same year and in 2007 the exportation income was higher (Table 1.4). The Northern region of Portugal, and more specifically the Trás-os-Montes area, is the major producer of chestnut fruits (18 071t), corresponding to 82.2 % of the total national production in an area of 25 975 ha, in the year of 2007 (Table 1.5).

Table 1.4 - Values of importation, exportation and commercial value of chestnut fruits in Portugal (INE, 2006 to 2009).

	2005		**2006**		**2007**		**2008***	
	IMP	**EXP**	**IMP**	**EXP**	**IMP**	**EXP**	**IMP**	**EXP**
Quantity (Tons)	1 328	5 131	809	8 638	1 449	7 774	- **	- **
Commercial value (1000 euros)	1 635	8.175	1 084	10 958	1 967	14 844	1 769	12 517

* preliminary data; ** data not available due to the implementation of a new simplified data collection system.

Table 1.5 - Area, production and income of chestnut fruits in the different agrarian regions of Portugal in 2007 (GPP, 2010).

Agrarian Regions	Area		Production		Income/ha	
	ha	% ha	t	% t	t/ha	% t/ha
Portugal	**30 301**		**21 990**		**0.73**	
Continent	**30 133**	**99.45**	**21 646**	**98.44**	**0.72**	**98.63**
North	26 444	87.27	18 508	84.17	0.70	95.89
Trás-os-Montes	25 975	85.72	18 071	82.18	0.70	95.89
Entre Douro e Minho	469	1.55	437	1.99	0.93	127.40
Centre	3 136	10.35	2 450	11.14	0.78	106.85
Beira Litoral	522	1.72	1 030	4.68	1.97	269.86
Beira Interior	2 614	8.63	1 420	6.46	0.54	73,97
Ribatejo e Oeste	14	0.05	12	0.05	0.86	117.81
Alentejo	533	1.76	671	3.05	1.26	172.60
Algarve	6	0.02	5	0.02	0.83	113.70
Autonomous Regions	**168**	**0.55**	**344**	**1.56**	**2.05**	**280.82**
Açores	97	0.32	281	1.28	2.90	397.26
Madeira	71	0.23	63	0.29	0.89	121.92

1.3.2 Percentage of Chestnut used in the Fresh and Frozen Markets

A considerable share of this production is sold to agro-industrial enterprises that use them for either the fresh or frozen markets. The major one of this sector in Portugal, and as referred before, is Sortegel - Produtos Congelados, S.A. (Sortes-Bragança), responsible for the commercialization of fresh chestnut fruits (19% of commercialized products) and frozen chestnut fruits (74% of commercialized products), and exporting 90% of the enterprise total production (Ferreira-Cardoso and Pimentel-Pereira, 2007). Approximately 7% of the fruits are lost either due to post-harvest storage diseases (from harvest point up to March/April) or damage during the industrial processing.

Unfortunately the transformation market of chestnut fruits in Portugal is almost nonexistent. However, in other countries such as Spain, France, Italy and Switzerland, products derived from chestnut fruit transformation industry (e.g. “marron-glacés”, tinned chestnuts and chestnut liquors) are highly appreciated and valued (Figures 1.7 and 1.8). Thus, we can assume that a great part of Portuguese exportations to these countries are used to produce these types of chestnut products.

Figure 1.7 - Chestnut products: liquors (left) and whole chestnuts in tins and jars (right).

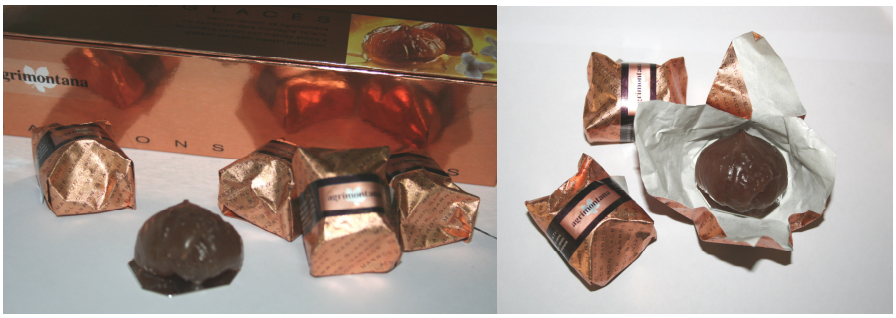

Figure 1.8 - "Marron glacés".

1.4 Commercial and Technological Characteristics

The chestnut market in Portugal is based on three types of commercialization: (1) fresh fruits sold directly to the consumers and used mainly in gastronomy (traditional recipes); (2) exportation of fresh or frozen chestnut fruits; (3) "transformed" fruits - a market almost nonexistent in Portugal, but if improved and supported would allow the use of the lower quality fruits leading to the production of a valued product by the consumers. Depending on the destination of the chestnut fruits, important characteristics are fundamental to determine the appropriate market: polyspermy, splitting, post-harvest storage, maturation period, ease of peeling, calibre, colour, indentations and flavour (Ferreira-Cardoso, 2007).

1.4.1 Calibre

The size of the chestnut fruits is one of the main commercial characteristics that have an influence on the type of utilization of the fruits. The calibre can be measured by the quantity of fruits that weigh 1kg, and this parameter is usually of high importance to the consumers. This characteristic is directly correlated with the edaphoclimatic conditions and the cultural treatments of the chestnut trees, being different between years. According to Ferreira-Cardoso (2007), the

Portuguese cultivars that present a better calibre (50 to 70 fruits/kg) are Judia, Martaínha, Lamela, Boaventura, Rebolão and Bebim.

1.4.2 Aspect (colour, shell splitting, fungal and pest infestation)

An attractive aspect is the result of characteristics such as a good colour and the absence of damage to the fruit (shell splitting or fungal/parasitic infestation). However, some cultivars may present these negative characteristics. Ferreira-Cardoso (2007) proposed that the appearance of the shell splitting is a genetic characteristic that may also be related to the occurrence of rain after the complete fruit and shell maturation, allowing the growth of the seed and causing the shell splitting. According to the same author this is more commonly observed in the more precocious cultivars and therefore allows infestation by insects and parasites leading to a poor storage capacity. The Portuguese cultivar that is the most sensitive to splitting is Aveleira (Ferreira-Cardoso, 2007).

1.4.3 Polyspermy

Polyspermy refers to the number of cotyledons/seeds inside the chestnut fruit. According to Bergougnoux *et al*. (1978) when the percentage of polyspermic fruits is less than 12% the variety is characterised as "marron", while if greater than 12% it is designated as "chestnut". This author stated that the "marron" chestnut fruits are highly appreciated either for commercialization as frozen kernels or for transformation ("marron-glacés") because they possess a better peeling, while the polyspermic fruits ("chestnuts"), because of their low ease of mechanical peeling, are consumed mainly as fresh and used to produce products such as creams, purees and flour. In general the Portuguese cultivars present a percentage of polyspermy of less than 12%, a characteristic that shows why they are well appreciated for industrial processing (Ferreira-Cardoso, 2007).

1.4.4 Type of Fruit Indentations

The type of fruit indentations directly affects peeling of the chestnut fruits, because the greater depth and number of indentations increases the difficulty of removing the integument from the kernel (Figure 1.2). This characteristic is intensely related to the type of cultivar and highly important for the selection of the best cultivars for the processing industry. Therefore the depth and number of indentations is decisive for successful industrial peeling. According to

Ferreira-Cardoso (2007) the Portuguese cultivars with the lowest number of fruits with indentations are Benfeita, Trigueira and especially Longal.

1.4.5 Ease of Peeling

The "ease of peeling" is the most important characteristic for the chestnuts destined for the industry, and this factor is dependant on the depth and number of indentations and polyspermy, as described previously. In general most of the Portuguese cultivars present a good peeling, particularly Longal (Ferreira-Cardoso, 2007).

1.4.6 Maturation

Ferreira-Cardoso (2007) stated that the maturation time is a characteristic that can be determined by the genotype of the pollen (masculine progenitor). According to this author, in general the cultivars that mature first are the ones that reach the fresh markets sooner and are therefore more expensive, but as described above these cultivars present a higher percentage of split shells and are more vulnerable to infestation by insects and fungi during storage.

1.4.7 Storage

The storage capacity of the chestnut fruits depends not only on the cultivar but also on the climatic conditions. Ferreira-Cardoso (2007) previously reported that in order to preserve the fruits during longer periods and to ensure optimum storage conditions, post-harvest treatments should be performed (removal of fruits infested with insects and contaminated by fungi before storage). Payne *et al*. (1983) stated that chestnut fruits can be safely stored up to 4 to 6 months at 0 ºC, and even up to 12 months if the water content is less than 40% and that the fruits are stored in refrigeration chambers with a relative humidity of 65-70%. The same authors stated that the appearance of condensation should also be avoided to prevent the growth of rot fungi.

1.4.8 Organoleptic Properties

Characteristics such as flavour (sweetness) and texture are directly related to the content of starch and soluble sugars present in the chestnut fruits (Saito *et. al.*, 1993) and are decisive, especially for the fresh market. In general the Portuguese cultivars are known and highly appreciated for their excellent organoleptic properties, particularly the cultivars Longal and/or Enxerta (Ferreira-Cardoso, 2007).

1.5 Industrial Processing - Frozen Market

1.5.1 Processing Stages

The chestnut fruits of Portuguese cultivars are usually harvested in October/November from orchards where they are mainly implanted. As previously explained the major company in the chestnut fruit production and processing sector in Portugal is Sortegel-Produtos Congelados S.A. (Sortes - Bragança). After arrival at the company the chestnut fruits can be commercialized through two market lines: fresh and frozen (Figure 1.9).

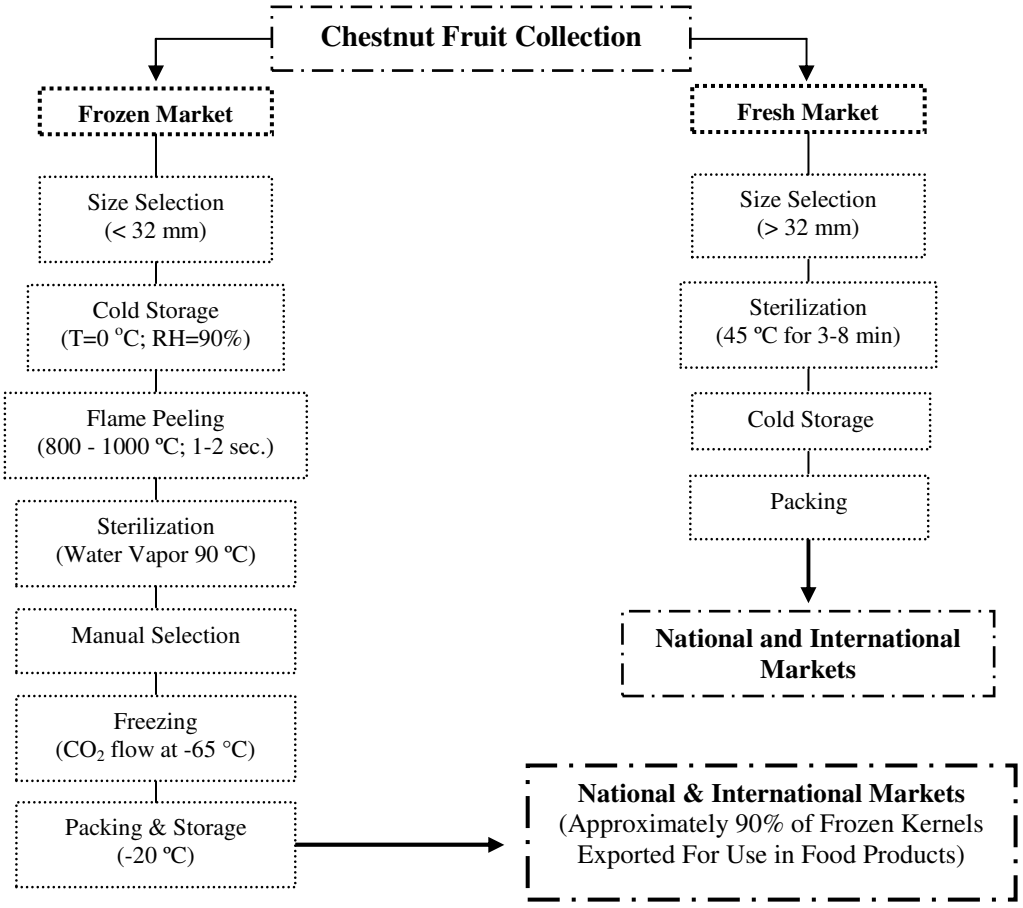

Figure 1.9 - Diagram representing the chestnut fruit processing.

Due to the large quantity of chestnut fruits acquired by Sortegel, part of the harvest is kept in large containers at room temperature (4-5 ºC), in a storehouse until they are processed. According to Borges *et al*. (2009) for the fresh market line first is performed a careful selection of the healthy fruits (visually free of pests and fungi) followed by a calibration that is done in a rotational tunnel with holes of specific diameter. The fruits commercialized fresh are usually

over 32 mm of size. The same authors stated that after calibration the chestnut fruits are disinfected/sterilized by immersion in a water bath at 45 ºC during 3 to 8 minutes, followed by forced ventilation. After these steps the chestnuts are stored in refrigerated chambers (during 1 to 2 months at 0-2 ºC), until packing for commercialization in national and international markets. In Sortegel the fruits selected for the frozen market line (usually under 32 mm of diameter) can be stored up to 6 months (at 0 ºC with relative humidity of 90%) before peeling by flame ("brûlage", at 800 to 1000 ºC during 1 to 2 seconds) in a rotary cylindrical oven. Thus the pericarp and integument are easily removed from the seed, followed by immersion in a water bath at 90 ºC (sterilization) and a manual selection of the kernels. These fruits are then frozen in a tunnel with a CO_2 flow at -65 ºC between 15 to 20 minutes, then packed and kept at -20 ºC until exportation or sold to the national market.

1.6 Chestnut Fruit Nutrient and Non-Nutrient Composition and Health Benefits

The economic and social importance of chestnut fruits certainly justifies the intense research made in the past few decades with the objective of increasing the appreciation and valorisation of these fruits. Also the increasing concern about human health means chestnut fruits could play an important role as part of a healthy diet rich in beneficial nutrients and antioxidant compounds with positive health effects. There have been a few studies on the composition of various nutrients and bioactives in the raw fruits (stage A in the processing scheme), but no analyses of the whole industrial process have previously been published. Chestnut fruits contain various nutrients (starch, free sugars, proteins, amino acids, lipids/fat, fatty acids), polyphenolics, vitamins, and minerals that are important for health; see table 1.6 for a summary of chestnut fruit composition. A detailed description of compounds previously found in chestnut fruits is presented in the review paper (Chapter 7).

In addition to their uses in human nutrition, Ferreira-Cardoso (2002) previously analysed the use of low calibre and commercial value chestnut fruits in the fattening of Duroc pigs (non-castrated males), showing that these nuts are a good energy source and a good food supplement contributing to the nutritional requirements of the pigs, but also improving the sensorial taste of the meat and ham. *Castanea sativa* wood is being used as source of tannins, as potential replacements for antibiotics in animal production, in order to improve other animal's health; increasing their life spans by preventing or eliminating the gut colonization of parasites (Zimmer and Cordesse, 1996; Schiavone *et al*., 2008).

From the industries responsible for the chestnut processing a large amount of waste material is generated (the inedible pericarp - 8.9 to 13.5% - and integument - 6.3 to 10.1%, that surrounds the edible fruit). It is of the utmost importance to reduce these residues that can cause environmental pollution. The antioxidants and other components present in shells could be used as additives for animal feeds and human foods, allowing the recycling and decrease of these vegetable wastes as well as becoming a potential economic source and thus increasing the competitiveness of the producers (research data on the composition of selected bioactives in pericarps and integuments of some Portuguese chestnut fruits is presented in Chapter 6).

Table 1.6 - Chestnut fruits nutrient and non-nutrient composition.

Component	**Value Range for Chestnuts Fruits***
Total Starch (g $100g^{-1}$ DM)	38.6 - 67.2
Fibre (g $100g^{-1}$ DM)	NDF (2.7 - 28.9); ADF (0.5 - 4.5); ADL (0.02 - 1.3); Cellulose (0.5 - 3.6)
Free Sugars (g $100g^{-1}$ DM)	Sucrose (6.6 - 29.7); Glucose (0 - 2.3); Fructose (0.04 - 2.3); Maltose (0 - 1.8)
Crude Fat (g $100g^{-1}$ DM)	0.8 - 4.4
Fatty Acids (g $100g^{-1}$ Total Lipids)	SFA (14.1 - 27.7); C18:1 (17.4 - 37.6); MUFA (17.9 - 39.3); C18:2 (37.6 - 50.9); PUFA (42.0 - 60.1)
Crude Energy (Kcal $100g^{-1}$ DM)	401 - 428
Crude Protein (g $100g^{-1}$ DM)	2.3 - 10.9
Free Essential Amino Acids (mg $100g^{-1}$ FW)	Arg (0.5 - 48.2); Ile (0.3 - 14.8); Leu (0.3 - 5.8); Phe (0.5 - 9.6); Thr (3.6 - 10.0); Trp (0.3 - 2.2); Val (0.8 - 11.5)
Major Minerals (mg $100g^{-1}$ DM)	Ca (26 - 72); P (68 - 305); K (473 - 1475.7); Mg (47.4 - 100); S (26.4 - 132.8); Fe (1.4 - 10.9)
Vitamin E (µg g^{-1} FW)	α-tocopherol (0.02 - 0.1); γ-tocopherol (3.8 - 27.3); δ-tocopherol (0.2 - 1.0)
Vitamin C (mg $100g^{-1}$ FW)	Ascorbic + Dehydroascorbic Acid (0.77 - 40.2)
Pigments (µg g^{-1} FW)	Lutein (0.1 - 0.6); β-Carotene (0.01 - 0.1); Total Minor Carotenoids (0.02 - 0.2); Lutein Esters (0.1 - 0.4); Total Carotenoids (0.23 - 1.30)
Phenolics (mg g^{-1} FW)	Total (0.7 - 36.7); Gallic Acid (2.8 - 25.6); Ellagic Acid (0.05 - 47.8)
Dry Matter (g $100g^{-1}$ FW)	35.6 - 59.7
Total Ash (g $100g^{-1}$ DM)	1.5 - 3.2

* Range values are from data presented in Chapter 7.

1.6.1 Starch and Sugars

According to Drozdowski and Thomson (2006) carbohydrates are a great part of the ingested food in our daily diet and they can be simple monosaccharides (glucose, fructose and galactose), disaccharides (lactose, sucrose) and complex polysaccharides such as starch and

fibre. Within the human diet carbohydrates constitute between 45 to 65% of the calorific intake (USDA, 2008b). These studies reported that most of these sugars are digested by glycosidases present in saliva and secreted from the pancreas into the small intestine, and they are further catabolized into monosaccharides by enzymes present in the brush border membrane of enterocytes lining the intestines.

Chestnut fruits are very rich in starch and also contain significant amounts of free sugars and fibre. Starch and sucrose are important in the assessment of the chestnut fruit commercial quality (Bernárdez *et al*., 2004; Attanasio *et al*., 2004). Previous analyses identified mono- and di-saccharides (glucose, fructose, sucrose and maltose) in chestnut fruits (Table 1.6). These mono- and di-saccharides are readily absorbed in the small intestine (Ferraris, 2001; Kellett *et al*., 2008). Studies in Spanish and Italian chestnut cultivars have shown high contents of starch and additionally studies have been performed on Italian chestnuts to evaluate the specific contents of the two forms of chestnut starch - amylose (33% of starch) and amylopectin (67% of starch) (Pizzoferrato *et al*., 1999; Attanasio *et al*., 2004; Pereira-Lorenzo *et al*., 2006). Starch is mainly digested into glucose in the small intestine by salivary and pancreatic α-enzymes and the resultant mixture of dextrins is hydrolyzed into glucose by two small-intestinal brush-border exohydrolases (maltase-gluocoamylase and sucrase-isomaltase) (Ao *et al*., 2007; Sim *et al*., 2008). The undigested (resistant starch) part together with dietary fibre undergoes fermentation in the large intestine, consequently producing short chain fatty acids (SCFA). Fässler *et al*. (2006a and 2006b) previously described four types of resistant starch: 1) physically inaccessible (partly milled grains or seeds); 2) native starch granules (green banana or raw potato); 3) retrograded starch (food processing); and 4) chemically modified starches. These authors stated that resistant starches are beneficial because of their role as substrates for colonic fermentation and high butyrate production (associated with the prevention of colon cancer). SCFA (short chain fatty acids) are important substrates in oxidative energy metabolism and signalling metabolites with a major impact on colonocyte proliferation and differentiation. In general SCFA decrease the colonic pH and consequently the conversion of primary to secondary bile acids and therefore the presence of carcinogens in the colon (Fässler *et al*., 2006a and 2006b). The resistant starches can be considered as prebiotics due to the fact that they provide a substrate (nourishment) to the bacteria present in the gastrointestinal tract (Czarnecki-Maulden, 2008). Therefore, starches are highly important and their digestion is increasingly associated with benefits for human health. The presence of less gelatinized starches in the mouth lowers the risk of cariogenesis (plaque formation) and in the upper gut the chemically modified starches may be used to transport probiotic organisms and therefore improve the immune response and

suppression of microbial pathogens (Bird *et al*., 2000). These modified starches can also be used to manipulate the gut bacteria and their products with benefits for the host health.

1.6.2 Fibre

The fibrous fraction, predominantly derived from plant cell walls and present in most plant foods, is a fundamental part of a healthy diet due to its role as a "prebiotic". Fibre functions as a growth substrate (carbon source) for the beneficial *Bifidobacteria* and *Lactobacilli* present in the intestinal tract (predominantly in the colon), which can lead to decreases in cholesterol levels, reduction in the risk of cardiovascular diseases, positive regulation of insulin response, increases in anticancer mechanisms (colon and rectal), and positive effects on the metabolism of blood lipids (Prosky, 2000). The fermentation of dietary fibre by the colonic bacteria leads to the production of short chain fatty acids (SCFA) and these have an important role in the maintenance of colonic integrity and metabolism, and have the potential of acting as therapeutic agents in important diseases such as various forms of colitis, antibiotic-associated diarrhoea and colon cancer (Cook and Sellin, 1998). Dietary fibre consists of lignin together with the polysaccharides that were not hydrolyzed by the digestive tract enzymes and include both hydrolysable and non-hydrolysable polymers (celluloses, hemicelluloses, lignin, pectins, gums and mucins) resistant to digestion. Previous studies have determined the dietary fibre content of European (*Castanea sativa*) and Korean (*Castanea mollissima*) chestnut fruits (Peña-Mendez *et al*., 2008; Lee *et al*., 2008). Other authors previously determined the content of fibre (NDF, ADF and ADL) in chestnut fruits (Table 1.6; Ferreira-Cardoso, 2002; Ferreira-Cardoso *et al*., 1993; Barreira *et al*., 2009a).

1.6.3 Crude Fat and Fatty Acids

Mono-unsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) are found in dietary nuts (e.g. almonds, hazelnuts and walnuts); in chestnut fruits they are present in lower levels (Table 1.6). MUFA and PUFA are known for their anticancer effects and their association with a decreased risk of development of cardiovascular diseases and neurological function disorders (Hardaman, 2002; Whelan and Rust, 2006). There are major health benefits from regular intake of omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFA) and these fatty acids are also being introduced in non-traditional food sources in order to fortify our diet with health promoting ingredients.

Portuguese chestnut cultivars have previously revealed low crude fat contents with low levels of saturated fatty acids and high levels of unsaturated fatty acids (Borges *et al*., 2007). The same authors stated that the major unsaturated fatty acids that can be found in fat/total lipids of chestnut fruits are oleic (C18:1), linoleic (C18:2) and α-linolenic (C18:3), constituting more than 85% of the total fatty acid content. According to Whelan and Rust (2006) the n-3 fatty acids (18-24-carbon with three or more double bonds) cannot be synthesised by the human organism since humans are not able to desaturate the n-3 or n-6 bonds, so they are obtained exclusively from the diet. The same authors stated that the major unsaturated fatty acid present in the diet is α-linolenic acid (ALA 18:3, n-3) from which all the n-3 fatty acids are derived. The major ALA dietary sources are soybean and canola oils as well as nuts, seeds, vegetables and fruits. Hardaman (2002) emphasized the beneficial effects of n-3 fatty acids for decreasing the risk of developing arteriosclerosis and breast, prostate and colon cancers. The same author stated that n-3 fatty acids can also slow the growth of cancer xenografts, increasing the efficacy of chemotherapy and decreasing its side effects.

1.6.4 Crude Energy

Any food source possesses an energetic value (crude energy) that is produced by the oxidative degradation of carbohydrates, proteins and lipids. Part of this energy is lost from the body through faeces, urine and as gases, so not all of the released crude energy (CE) is available to the organism. The remaining energy that is available to the metabolic mechanisms allows the maintenance and thermoregulation of the organism. Only a few analyses of the crude energy in chestnut kernels have been done and these revealed significant values (Table 1.6) showing that the chestnut fruits can be used as an important source of dietary energy (Barreira *et al*., 2009a).

1.6.5 Crude Protein and Amino Acids

Proteins (both quantity and quality) are an important part of a balanced diet. They participate in most processes within cells e.g. enzymes that catalyze biochemical reactions and are vital for various metabolic processes. Others have structural or mechanical functions e.g. the proteins in the cytoskeleton. The cytoskeleton form a system of scaffolding that maintains cell shape. Proteins are also important in cell signaling, immune responses, cell adhesion, and the cell cycle. Humans and animals cannot synthesize all the amino acids necessary and they must obtain various essential amino acids from the diet. Through digestion, the ingested proteins are

catabolized by proteases, in the stomach and the small intestine, into free amino acids that are then absorbed in the small intestine and used for various metabolic processes.

Chestnut fruits contain between 2.3-10.9g $100g^{-1}$ DM of crude protein (Table 1.6). There have been various studies on the amino acid composition of chestnut proteins (see paragraph below) and in addition it has been shown that chestnut fruits do not contain glutens. Celiac disease is a polygenic disorder caused by intolerance to peptides derived from digestion of the cereal protein gluten (Green and Jabri, 2006). The alcohol-soluble fraction of gluten (gliadin) is toxic for the sufferers of this disease and 40% of the risk of this disease is associated with the presence of the HLA (human leukocyte antigen) genes DQ2 and DQ8, that are carried by 30-40% of Caucasians but less than 3% of this percentage will develop celiac disease (Louka and Sollid, 2003). In consequence, sufferers can only use a gluten-free diet. A very good alternative to cereals is chestnut fruits because they do not contain gluten and there are many new products derived from chestnuts and chestnut flour that have been created to replace the wheat/cereal derived foods.

The amino acids present in the dietary proteins, are also of high importance and can be divided in two classes: essential amino acids (the ones that the human body does not synthesize and are obtained exclusively from the diet) and non-essential amino acids (synthesized by the human body in adequate concentrations). Total amino acids (including free and protein-derived) have been previously analyzed in chestnut fruits from various cultivars revealing that the predominant protein-derived amino acids are aspartic acid and glutamic acid (Desmaison *et al*., 1984; Meredith *et al*., 1988; Borges *et al*., 2008).

In European chestnut fruits (*Castanea sativa*) the non-protein amino acid γ-amino butyric acid (GABA), derived from the amino acid arginine, has also been found with a range between 50 to 236mg $100g^{-1}$ DM (Tixier and Desmaison, 1980). GABA is an important inhibitory neurotransmitter of the central nervous system found mainly in the brain but also in the spinal cord, where about half of the inhibitory synapses use GABA (Tortora and Grabowski, 2000). GABA is synthesized from glutamate catalyzed by the enzyme L-glutamic acid decarboxylase which uses pyridoxal-phosphate (active form of vitamin B6) as its cofactor, converting glutamate (main excitatory neurotransmitter) into GABA (main inhibitory neurotransmitter) (Desmaison and Adrian, 1986). These authors also stated that a common consequence of the lack of vitamin B_6, and the associated poor synthesis of GABA, is convulsions in children. GABA has a calmative effect in the body by decreasing the neuron activity and in combination with niacinamide and inositol prevents anxiety-related messages from reaching the motor centres of the brain by blocking the receptor sites (Korel and Balaban, 2009). Other authors reported that

GABA is also synthesised by many bacteria, especially *Lactobacilli*, and may be involved in the maintenance of neural development and activities, and thus increasing host health (Forsythe *et al*., 2009).

1.6.6 Minerals

Minerals are involved in many essential biological functions, therefore a healthy nutrient-rich diet is an important topic of public health and this includes a balanced mineral intake (FAO/WHO, 2004). Among the vast metabolic functions of minerals some can be highlighted. For example, calcium confers rigidity to the skeleton, playing an important role in signal transduction pathways, in contraction of all muscle cell types and also acting as an enzyme cofactor. Selenium is implicated in protection of body tissues against oxidative stress, maintenance of defences against infection and modulation of growth and development. Magnesium (30-40% present in muscles and soft tissues) is often a cofactor of enzymes involved in energy metabolism, protein synthesis, RNA and DNA synthesis and maintains the electrical potential of nervous tissues and cell membranes. Zinc is also an essential mineral, performing an important role as part of the active site of over 300 enzymes that participate in the synthesis and degradation of carbohydrates, lipids, proteins, and nucleic acids, also playing an important role in polynucleotide transcription (genetic expression) and in the immune system. Iron functions as an integrated part of enzyme systems and as component of haemoglobin in red blood cells is the transporter of oxygen through the body. It is also an essential constituent of the cytochromes that are involved in the electron transport chain within cells and co-factor of important xenobiotic-metabolizing/anticancer enzymes e.g. cytochrome P450-containing enzymes (FAO/WHO, 2004). The genotype, weather conditions and the mineral composition of the soil are the main decisive factors determining the content of minerals found in the chestnut fruits. These compounds were previously analyzed in chestnut fruits revealing important macro-elements (Ca, P, K, Mg and S), with potassium representing the majority, and also important micro-elements (Fe, Cu, Zn and Mn) (Table 1.6; Ferreira-Cardoso, 2002; Pereira-Lorenzo *et al*., 2006; Borges *et al*., 2008).

1.6.7 Antioxidant and Other Vitamins

Vitamins are organic compounds, required in relatively small quantities, and cannot be synthesized in sufficient quantities by organisms i.e. they can only be obtained from the diet. In many cases fruits, nuts and vegetables are very good sources of vitamins. They have diverse functions including antioxidants (vitamins C and E), regulators of tissue and bone growth

(vitamin A), regulation of mineral metabolism (vitamin D), and co-factors of enzymes (many of the B vitamins have this function). Vitamins are divided into two classes - the water-soluble and the fat soluble. For humans there are 13 essential vitamins: nine are water-soluble (vitamin C and 8 B vitamins) and four are fat-soluble (A, D, E and K). The reduced form of vitamin C (ascorbic acid or ascorbate) is an essential health compound that humans cannot synthesize, so it is exclusively obtained from the consumption of fruit and vegetables. This vitamin is an electron donor (reducing agent or antioxidant) and the biochemical and molecular role is mainly as an antioxidant, as part of a redox cycle with dehydroascorbic acid. Two important consequences of vitamin C deficiency are the diseases scurvy and anaemia (FAO/WHO, 2004; Ching and Mohamed, 2001). Another important vitamin and the major cell lipid-soluble antioxidant is vitamin E, also only obtained from food, and its main biological role is to protect the polyunsaturated fatty acids (PUFA), low-density lipoprotein (LDL) and other cell membrane components from oxidation by free radicals. The functions displayed by this vitamin are associated with reducing the risk of cancer and cardiovascular diseases. Previous studies reported the compositional content of these vitamins in chestnut fruits (Table 1.6; Peña-Mendez *et al.*, 2008; Barreira *et al.*, 2009b). Other important vitamins previously reported in raw chestnut kernels are: vitamin A (retinol - visual system, growth, epithelial cellular integrity, immune function and reproduction), thiamine (B_1 - coenzyme in carbohydrates and branched-chain amino acids metabolism), riboflavin (B_2 - coenzyme in redox reactions), niacin (B_3 - precursor of important co-factors of enzymes and proteins in the electron transport chain i.e. NAD^+/NADH and $NADP^+$/NADPH), pantothenic acid (B_5 - constituent of coenzyme A and involved in fatty acid metabolism), pyridoxine (B_6 - coenzyme in amino acids, glycogen and sphingolipid metabolism) and folic acid (B_9 - is an important co-factor of enzymes involved in various metabolic pathways, in the formation of red blood cells, in DNA synthesis and repair, and may also be important in various neurological functions) (FAO/WHO, 2004; Korel and Balaban, 2009).

In chestnut fruits only the carotenoids beta-carotene (pro-vitamin A), lutein and zeaxanthin have been found (no chlorophylls or related derivatives have been reported) (USDA, 2008a). Beta-carotene has been used in the treatment of diseases such as erythropoietic protoporphyria, a light-sensitive condition, and may have positive effects that enhance immune cell responses. The other two carotenoids, lutein and zeaxanthin, are also associated with the protection of the retinal pigment epithelium against the oxidative effects of blue light (ultra-violet) (FAO/WHO, 2004). Carotenoids also provide other benefits for human health such as redox sensitive cell signalling and vitamin A signalling pathways (Elliot, 2005).

1.6.8 Secondary Metabolites (Phytochemicals)

Phytochemicals (plant secondary metabolites) are non-nutrients. There are many different structural classes of phytochemicals but those that have been investigated in greatest detail and are important for human health include (poly)phenolics such as flavonoids and tannins, sulphur compounds such as glucosinolates and *Allium* species (poly)sulfides and sulfinates, and nitrogenous compounds such as alkaloids and non-protein amino acids (Kashiwada, 1992; Haslam, 1996; Kuttan, 2000; Herman-Antosiewicz and Singh, 2004). Phytochemicals do not provide any energy for the organism. However, in the last few decades many classes of phytochemicals have been shown to have positive effects on human health e.g reducing the risk of various diseases, such as cancers and coronary heart disease, through the activation of signal transduction pathways, antioxidant mechanisms, and xenobiotic metabolizing enzymes (Moreno *et al*., 2006; Espín *et al*., 2007; Stevenson and Hurst, 2007; Kok *et al*., 2010). Therefore their identification and quantification in animal and human foods is very important. There have been various studies on evaluating the composition of phytochemicals in chestnut fruits and other tissues.

1.6.8.1 Phenolics and Polyphenolics

Simple phenolics, flavonoids and more complex tannins (condensed, gallotannins and ellagitannins) are associated with various positive health benefits such as antioxidant effects, decreases in the risk of cardiovascular diseases, anticancer mechanisms and anti-inflammatory properties (Veluri *et al*., 2006; Mertens-Talcott *et al*., 2006; Hooper *et al*., 2008). There are very few studies on the chestnut fruits content of phenolics (gallic and ellagic acid) (Table 1.6; Barreira *et al*., 2008). Low levels of condensed tannins (procyanidins) have previously been reported in chestnut fruits (Pascual-Teresa *et al*., 2000; Gu *et al*., 2004). More details on the composition of phenolics in chestnut fruits and other tissues are presented in the review paper (Chapter 7).

1.6.8.2 Alkaloids

Alkaloid-rich plants have been used for thousands of years in traditional medicine and are known to have important medicinal effects in humans; currently many of the most important drugs are alkaloids or derived from alkaloids. (Wink, 1998; Schmeller and Wink, 1998; Aniszewski, 2007a and 2007b). The function of alkaloids in plants is still not clear, but in the past few decades experimental data (including “knock-out” mutants of different plant species

that are unable to synthesize alkaloids) strongly supports the theory of their role in defence mechanisms, especially against insect pests and mammalian herbivores (Wink, 1999). A previous study on *Castanea sativa* fruits identified a new pyrrole alkaloid, methyl-(5-formyl-1*H*-pyrrole-2-yl)-4-hydroxybutyrate (Hiermann *et al*., 2002). The biological/biochemical properties of this alkaloid in animals and humans are currently unknown.

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CHAPTER 2

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 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 being predominant among hydrolysable 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 analysed indicates a significant effect of this factor in practically all the constituents. On the other hand, the two-way analysis of variance shows that, besides the residual, the processing step and the interaction cultivar x processing step were the factors that more contributed for the total variation observed in the constituents analysed, 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.

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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2.1 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 agro-forestry systems, particularly at present (Bounous, 2005). 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" (Gabrielli, 1994). *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) (Manos *et al*., 2001). 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, United Kingdom and Switzerland; the first four countries are responsible for 91.5% of the exports of this nut from Portugal. In the last few years the demand for Portuguese chestnuts in the USA has increased, but the import levels have not reached those of the major importers listed above (GPPAA, 2004). In former times chestnuts were used as feedstuffs and as substitute for potatoes and wheat flour. Nowadays, apart from boiling/baking and roasting the utilization of chestnuts in Europe is for marron glace (Brouk, 1975).

Chestnuts are found in three major geographical areas: Asia, where *Castanea crenata* Sieb. and Zucc. (in Japan), *C. molíssima* Bl. (in China and Korea), *C. seguinii* Dode, *C. davidii* Dode, *C. henryi* Rehder and Wilson (Skan) (in China) thrive; North America, where *C. dentata* Borkh., *C. pumila* (L.) Mill., *C. floridana* Ashe (Sarg.), *C. ashei* (Sudw.) Ashe, *C. alnifolia* Nutt., *C. paucispina* Ash. predominate; and Europe, where the sweet chestnut (*Castanea sativa* Mill.) is predominant (Bounous, 2005).

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 (Ferreira-Cardoso *et al*., 1993; Pereira-Lorenzo *et al*.,

2006). Other researchers analyzed the lipids and fatty acid composition of chestnut kernels (Ferreira-Cardoso *et al*., 1999; Borges *et al*., 2007). There have been some published studies on the free amino acids content of chestnuts (Desmaison *et al*., 1984; Desmaison and Tixier, 1984). There have been several studies on the phenolic composition of chestnut wood and leaves but very few studies on kernels (Ferreira-Cardoso *et al*., 1988), and primarily for single components, e.g. the procyanidins (Lampire *et al*., 1998; Pascual-Teresa *et al*., 2000; Calliste et al., 2005).

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 would then be used to as part of ongoing research for improving processing methods and selection of *Castanea sativa* cultivars for breeding programs and optimizing health benefits of chestnuts.

2.2 Materials and Methods

2.2.1 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. Due to poor kernel yields during the harvest year the cultivar Martaínha was not industrially processed, and thus samples at stages B, C and D were not available. The samples were collected at the end of each processing step: A) fresh; B) after storage for 2 months at ±2 ºC; C) after industrial steam peeling at SORTEGEL and D) after freezing with liquid air and -20 ^{o}C storage at SORTEGEL (Figure 2.1). After the collection of each sample of the three cultivars at the respective processing stage, six sub-samples were taken and stored in a refrigerator at ±2 ºC (for samples from stages A, B and 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 ºC until analyzed. The samples were processed either by air-drying or freeze-drying depending on the analyses to be performed.

2.2.2 Chemicals

All chemicals and reagents were of analytical grade and obtained from various commercial sources (Sigma/Aldrich, Merck and Pronalab). All solvents were of HPLC grade and

all water was ultra-pure. All amino acid and phenolic standards were obtained from Sigma-Aldrich. For all standards HPLC calibration curves were constructed by injection of 20µL of different stock concentrations of the standards.

2.2.3 Processing Samples for the Different Analyses

Samples were stored unshelled at ± 2 ºC until analysis for a maximum of 3 days. The shells and pellicle were manually removed (stages A and B; Figure 2.1).

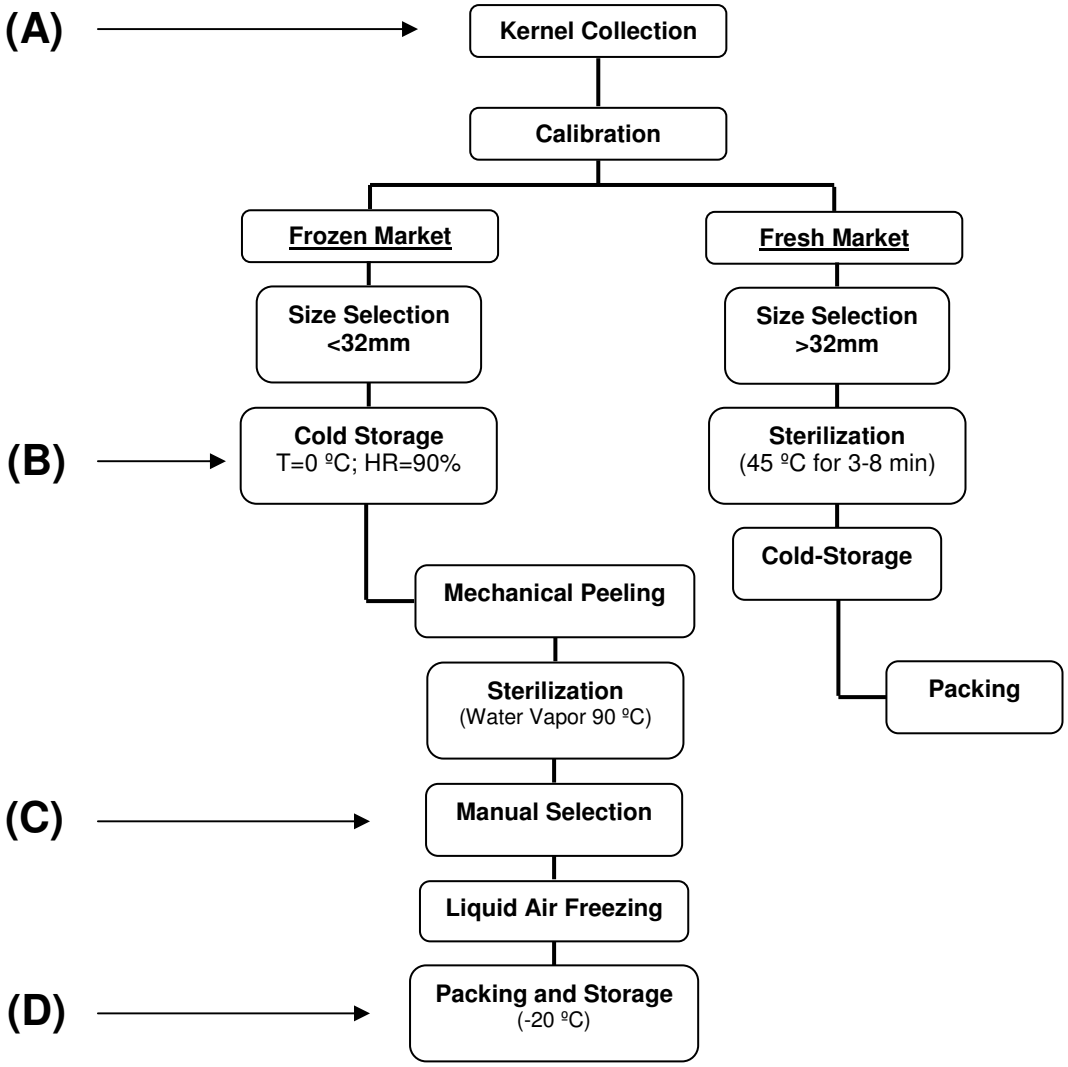

Figure 2.1 - Schematic representation of the industrial processing steps for frozen and fresh chestnut kernels.

2.2.4 Dry Matter, Organic Matter and Ash Contents

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 ºC until constant weight (for at least 24 h), to determine dry matter and for use in various primary metabolite analyses (Ferreira-Cardoso *et al*., 1999). Sub-samples of the air-dried samples (2.5g) in duplicate for each sample were analyzed for residual water content and ash content using previously validated methods (AOAC, 1975). In the first step, samples were submitted to a drying processing in a model USM/ULM 500 oven at 105 ºC (Memmert, Schwabach, Germany) for 12 hours, and then the samples were weighed. Dried samples are incinerated in a Furnace model 6000 high temperature oven (Thermolyne Corporation, Dubuque, USA) for 3 h at 550 ºC, and the ash content was obtained.

2.2.5 Extraction and Quantification of Crude Fat

For determination of crude fat contents sub-samples of the air-dried samples (3g), 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 constitutes the crude fat (AOAC, 1990).

2.2.6 Extraction and Quantification of Crude Protein

Sub-samples of the air-dried samples (1g) 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 (AOAC, 1990). The crude protein content was calculated by using 5.3 as the conversion factor, according to McCarthy and Meredith (McCarthy and Meredith, 1988).

2.2.7 Extraction and Quantification of Starch

Sub-samples of the air-dried samples (0.05g) in triplicate for each sample were analyzed for Starch. The method used starts with a conversion of the starch to glucose during two stages of an enzymatic treatment that is followed by a colorimetric determination of the glucose using a glucose-specific coupled enzyme reaction and chromogen system (Rasmussen and Henry, 1990). The method involves the initial enzymatic treatment of the finely powdered plant material with α-amylase using a previously described method (Salomonsson *et al.*, 1984). During this processing step initial breakdown of the starch to dextrins and oligosaccharides occurs, 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 involves the coupled enzymatic glucose oxidase/peroxidase reaction in combination with the 4-amino-antipyrine chromogen system (Trinder, 1969; Blakeney and Matheson, 1984).

2.2.8 Extraction and Quantification of Fiber (NDF, ADF, ADL)

The air dried samples were weighed (1.0g for NDF and 0.8g for ADF and ADL) in duplicate and the respective quantification of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined by the Van Soest detergent system (Robertson and Van Soest, 1981). Because of the starchy nature of samples, α-amylase a thermo-resistant enzyme (Sigma Chemical Co., Spruce Street, St. Louis, USA) was also used to enzymatically transform the chains of amylose and amylopectin to make them more susceptible to extraction from the fiber fraction.

2.2.9 Extraction and HPLC Analysis of Free Amino Acids

The freeze dried samples were weighed in triplicate (0.2 g) into 15mL 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 (Gehrke *et al*., 1987); essentially samples were loaded into plastic columns (Chromabond of Macherey-Nagel) containing 1mL 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 ºC with an air flow vacuum pump, and the evaporated fractions were re-suspended with 300µL ultra-pure water and then filtered (Whatman general purpose 0.2µm filter) and stored in vials in the refrigerator until HPLC analysis. The analysis was performed using a 150 x 4.6mm, 3 µm C18 Spherisorb S3 ODS2 reverse-phase column (Waters, USA) in combination with a Gilson HPLC system (Wisconsin, USA) 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 ºC. Data was processed using the Uni-Point software. The solvents were A (350mM disodium hydrogen phosphate with 250mM propionic acid (1:1), acetonitrile and ultra-pure water, 40:8:52) and B (acetonitrile,

methanol, ultra-pure water, 30:30:40); both solvents were filtered (0.2µm) 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.

2.2.10 Extraction and Quantification of Total Phenolics

Sub-samples of the freeze dried chestnut kernel samples (3 x 40mg) were extracted with 1mL of 70% methanol at 70 ^{o}C for 30 min in 2mL screw-top micro-tubes, using a vortex mixing every 5 min to optimize extraction. The samples were centrifuged (17,000*g*, 4 ^{o}C, 20 min) and sub-samples of the supernatant (3 x 100µL) 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 100µL instead of 50µL (Javanmardi *et al*., 2003). Gallic acid calibration curves were produced each week.

2.2.11 Extraction and HPLC Analysis and Quantification of Free Gallic and Ellagic Acids

Sub-samples of the freeze dried chestnut kernel samples (3 x 40mg) were extracted, using 950µL of 70% methanol with 50µL 1mg/mL naringin (in 100% methanol) as the extraction standard, at 70 ^{o}C for 30 min in 2mL screw-top micro-tubes. Tubes were vortex mixed every 5 min to optimize extraction. The samples were centrifuged (17,000*g*, 4 ^{o}C, 20 min) and filtered (0.2µm general purpose filter), and HPLC analyses were performed using methods previously described (Bennett *et al*., 2006); A 250 x 4.6mm, 5µm Luna C_{18} (2) main column, with a Securityguard pre-column (Phenomenex, UK) with a C_{18} cartridge in combination with a Thermo-Finnigan Surveyor HPLC system (solvent degasser, quaternary pump, thermostatically-controlled auto-sampler set at 10 ^{o}C, thermostatically controlled column oven set at 25 ^{o}C, a photodiode array detector set to collect overall data from 200-600nm and selected wavelengths of 227, 270, 370 and 520nm). Peak identifications were confirmed from retention time, UV-spectroscopic data and direct comparison to pure standards.

2.2.12 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 the processing steps evaluated (A - fresh; B - after storage; C - after industrial steam peeling; 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 analysis of variance. In this case, cultivars and processing steps were the sources of variation considered, having as error term the fixed effect of these factors. The contribution of each one of the sources (cultivar, processing step and their interaction cultivar x processing step) for the total variation observed was measured as described by some authors (Snedecor and Cochran, 1980; Sokal and Rohlf, 1981).

2.3 Results and Discussion

For each of the different analyses both one-way and two-way analyses of variance (ANOVA) 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 the all processing stages (A, B, C and D).

Previous studies have shown that the moisture content of chestnut kernels was between 40.3-60.1% of fresh weight (Pereira-Lorenzo *et al*., 2006). The results of the current research show that all three cultivars had high moisture contents but low ash contents (Tables 2.1 and 2.2). 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 2.2). There was a significant difference in the ash contents of the three cultivars, probably due to differences in cultivation conditions (Table 2.1).

The values for crude fat content previously reported for chestnut kernels were between 1.7-4.0% of dry matter (Pereira-Lorenzo *et al*., 2006). 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 2.1) 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 2.1); there were no significant differences in crude fat contents among the three cultivars. For the remaining processing steps the highest

values found for crude fat was 2.6% of dry matter for both Longal and Judia cultivars in the processing step C (Table 2.2). Recent analyses of 17 sweet chestnut cultivars showed values for crude fat from 1.67-3.50% of dry matter; lowest in cv. Lada (PDO Padrela) and highest in cv. Aveleira (PDO Terra Fria), respectively (Borges *et al*., 2007). Chestnut crude fat is predominated by three unsaturated fatty acids, oleic (C18:1), linoleic (C18:2) and alpha-linolenic (C18:3) acids, which account for more than 85% of the total fatty acid content (Borges *et al*., 2007). Mono- and poly-unsaturated fatty acids (MUFA and PUFA) have been associated with positive health effects. There are reviews giving dietary recommendations for omega-3 fatty acids, including alpha-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid to achieve nutrient adequacy and to prevent and treat cardiovascular diseases (Harris, 2004; Gebauer *et al*., 2006). The cardio-protective benefits of omega-3 fatty acids may be attributed to multiple physiological effects on lipids, blood pressure, vascular function, cardiac rhythms, platelet function, and inflammatory responses (Engler and Engler, 2006). In addition, there is data suggesting that both omega-3 and omega-6 PUFA have positive health effects in humans (Woodside and Kromhout, 2005).

In a previous study the cultivar Longal grown in Galicia had a value of 5.2% crude protein (Pereira-Lorenzo *et al*., 2006). The current analysis found that all three cultivars had low crude protein contents (Table 2.1) 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 2.1). 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 2.2). Previous studies have found that the crude protein content of chestnut kernels was between 4.5-9.6% of dry matter, using 5.3 as a multiplicative factor (Pereira-Lorenzo *et al*., 2006). There was a significant difference in crude protein contents of fresh kernels of the three cultivars, with Martaínha having the lowest content (Table 2.1).

Previous studies have shown that the starch content of chestnut kernels was between 42.2-66.6% of dry matter (Pereira-Lorenzo *et al*., 2006). The cultivar with the highest starch content was Longal grown in Galicia (Pereira-Lorenzo *et al*., 2006). 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 2.1). 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 2.2). There was no significant difference in the total starch contents of the three cultivars (Table 2.1). 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 (Pizzoferrato *et al*., 1999). It is known that the chestnut fruit store a reserve in the form of starch in cotyledons, and the content is three to four-fold higher than found in other nuts (Ensminger *et al*., 1995). The average value for starch content found in this study is slightly higher than those obtained by others researchers (Pereira-Lorenzo *et al*., 2006) who reported 57% starch relative to dry weight, and higher than the value obtained by a previous work (Ferreira-Cardoso *et al*., 1993) for the Portuguese cultivar Longal (53-55%).

The three main plant fiber fractions are generated by detergent and acid treatments (Van Soest and Moore, 1965). The first fraction is generated by treating the sample with a neutral detergent creating two fractions: cellular contents [neutral detergent soluble (NDS)] that include protein, sugars, starch, lipids, non-protein nitrogen, pectin, fructosans, water soluble minerals and vitamins; and cell wall components, representing the total fibrous fraction, that consists of cellulose, hemicelluloses, lignin, silica, keratin, waxes, cutin, insoluble minerals, lignified nitrogen compounds and are expressed as neutral detergent fiber (NDF) (Van Soest and Moore, 1965). Treatment of the sample with acid detergent produces two fractions: all the NDS components and also hemicelluloses (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 are expressed as Acid Detergent Fiber (ADF) (Van Soest and Moore, 1965). 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 Acid Detergent Lignin (ADL) (Van Soest and Moore, 1965). The values for NDF and ADF contents found in previous chestnut kernel analyses were between 9.4-28.5% and 2.3-4.5% of dry matter, respectively (Pereira-Lorenzo *et al*., 2006). 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%, 0.2, 0.2 and 0.3% of dry matter in fresh kernels of Martaínha, Longal and Judia, respectively, in processing stage A (Table 2.1). 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%, 0.5 and 0.4% of dry matter (Table 2.2). There were no significant differences for each fiber fraction among the three cultivars (Table 2.1).

Free amino acids in plant foods are generally divided into two classes, essential and non-essential. Essential amino acids are those that cannot be synthesized *in vivo* by humans, and 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 2.3 and 2.4). In total, fifteen 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, Thr) were only present in trace amounts (Table 2.3 and 2.4). Trace values of amino acids means 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/100g 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 2.3). 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 2.4). 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 2.4). The values found for the cultivars Judia and Longal, respectively, were for processing step D: aspartic acid (41.1 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 2.4).

Some studies analyzing the changes in the free amino acid composition of ripening chestnut seeds (Desmaison *et al*., 1984) at the maximum stage of maturity (mg/100g 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 (Desmaison and Tixier, 1984) studied the composition of free amino acids in several French chestnut cultivars and the range of values (mg/100g FW) found were: 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 compared with the other two cultivars (Tables 2.3 and 2.4).

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 2.1). 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 2.2). 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 (Calliste *et al*., 2005). Several species within the Fagaceae (including *Aesculus*, *Betula*, *Castanea*, *Castanopsis*, *Fagus* and *Quercus*) have very high tannin content 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 (Krisper *et al*., 1992) and procyanidins (Pascual-Teresa *et al*., 2000) 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 2.2). 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 2.1). 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 2.2). 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 (Bianco *et al*., 1998; Häkkinen *et al*., 1999; Häkkinen and Törrönen, 2000; Mullen *et al*., 2002; Priyadarsini *et al*., 2002; Mullen *et al*., 2003; Pastrana-Bonilla *et al*., 2003; Lee and Talcott, 2004; Janisch *et al*., 2006; Mertens-Talcott *et al*., 2006; Veluri *et al*., 2006). Free gallic acid was present in all three *Castanea* cultivars (Table 2.1). Martaínha had the highest free gallic acid content (9.1 mg/g FW). Free ellagic acid (Tables 2.1 and 2.2) was found in the chestnut kernels, as well various ellagitannins and procyanidins that were not determined in the present study.

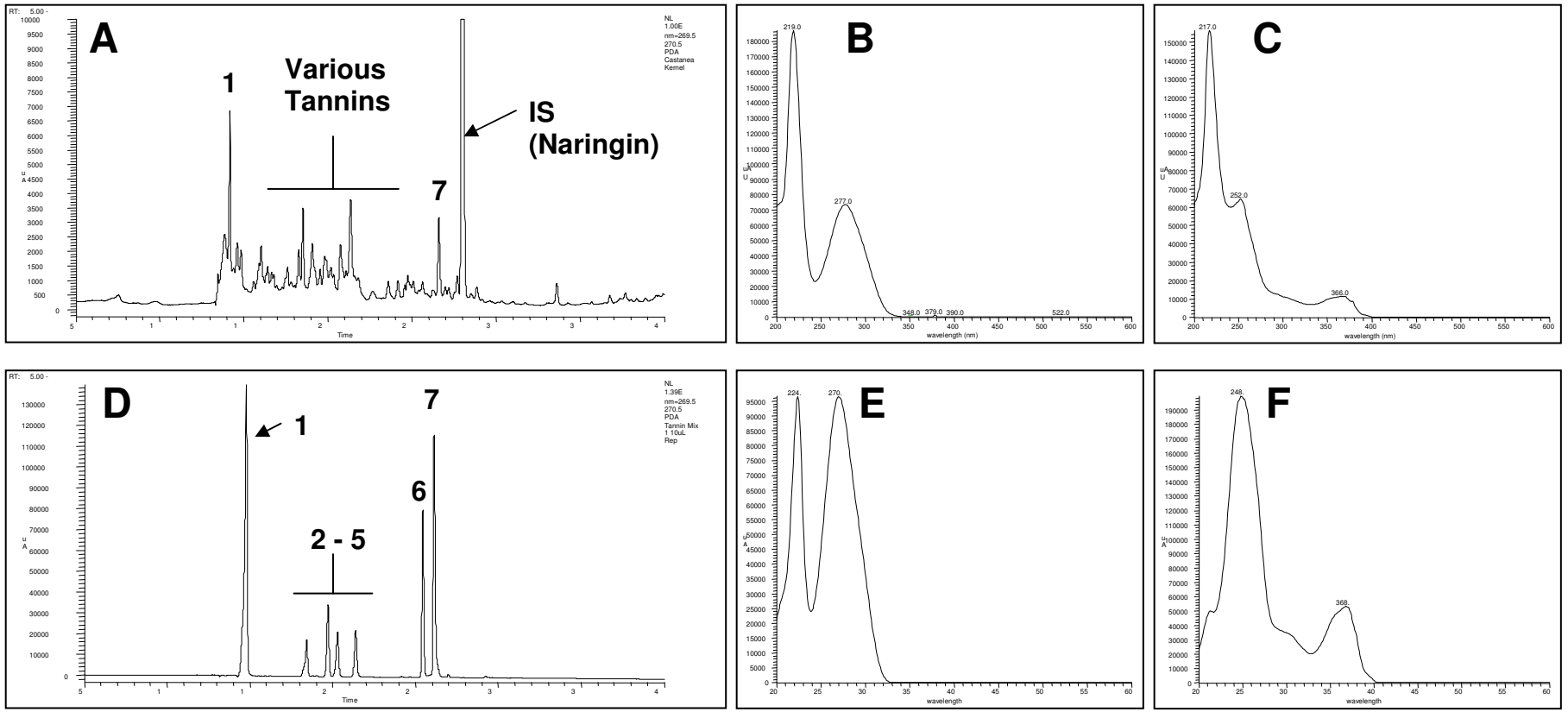

Figure 2.2 - Chromatograms and UV-visible spectra of a *Castanea* kernel phenolic extract and standards: *C. sativa* extract, 20µL injection (2A) and a 10µL injection of a 0.1mg/mL each phenolic standard mixture (2D). Spectra for gallic acid and ellagic acid in the samples (2B and 2C respectively) and in the standard mixture (2E and 2F 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. N.B. 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.

Previous studies on hardwood samples had found values of 19-89 mg/g of hardwood of total ellagic acid (Bianco *et al*., 1998); European chestnut had the highest value of 89 mg/g total ellagic acid (Bianco *et al*., 1998). 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 2.1). 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 2.2). There is good evidence for the positive health effects of gallic and ellagic acids, especially their antioxidant activities, positive effects on cardiovascular functions, anti-carcinogenic activities and anti-plasmodial activity (Banzouzi *et al*., 2002; Mullen *et al*., 2002; Priyadarsini *et al*., 2002; Pastrana-Bonilla *et al*., 2003; Lee and Talcott, 2004; Okuda, 2005; Janisch *et al*., 2006; Mertens-Talcott *et al*., 2006; Paivarinta *et al*., 2006; Veluri *et al*., 2006).

The one-way analysis of variance 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 2.1 and 2.3).

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 the constituents analysed (Tables 2.2 and 2.4). 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, in order 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 analysis of variance (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 non-controlled 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 x 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 i.e. it is known the cooking chestnuts affects the physico-chemical properties of starch but not total starch content (Pizzoferrato *et al*., 1999). Therefore, further studies need to be performed using more sensitive assays e.g. (SDS) PAGE for proteins, LC-MS and GC-MS 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.

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Table 2.1 - Basic chemical composition (g/100g DW) and phenolics (mg/g FW) of fresh (processing step A) raw shelled chestnut kernels from three cultivars[(a)].

Parameters	Judia	Longal	Martaínha	SL
Dry Matter[(1)] ($g\ 100g^{-1}$ FW)	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 (N x 5.30)	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 [(2)]	21.10±5.11 [b]	15.80±5.69 [a]	22.69±8.39 [b]	**
Gallic Acid [(3)]	4.30±1.52 [a]	3.46±1.72 [a]	9.07±3.43 [b]	***
Free Ellagic Acid [(3)]	4.84±1.37 [b]	2.71±0.70 [a]	9.64±3.39 [c]	***

[(a)] Tabulated values are sample means ± standard deviation (SD) of mean.

[(1)] Data is presented as mean ± SD g/100g FW.

[(2)] Data is presented as mean ± SD mg gallic acid equivalents / gram fresh weight.

[(3)] Data is presented as mean ± SD mg phenolic / gram fresh weight.

SL - Significance Level.

ns - not significant ($P>0.05$); *, **, *** - significant at $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

Within each row, means with a different letter are significantly different.

Table 2.2 - Basic chemical composition (g/100g DM) and phenolics (mg/g FW) of raw shelled chestnut kernels from two cultivars on the four processing steps analysed [(a)].

	JUDIA					LONGAL				
Parameters	**A**	**B**	**C**	**D**	**SL**	**A**	**B**	**C**	**D**	**SL**
Dry Matter [(1)]	50.37±1.54 [ab]	54.57±1.28 [c]	51.30±2.07 [b]	49.24±0.41 [a]	***	53.87±3.83 [b]	49.32±0.90 [a]	51.10±0.85 [a]	49.51±1.42 [a]	**
Crude Fat	1.72±0.39 [a]	1.94±0.70 [a]	2.58±0.16 [b]	2.47±0.07 [b]	**	1.56±0.31 [a]	1.56±0.57 [a]	2.58±0.13 [b]	2.35±0.28 [b]	***
Crude Protein (N x 5.30)	4.87±0.33 [a]	5.21±0.20 [b]	5.13±0.20 [ab]	5.20±0.25 [b]	ns*	5.13±0.43 [a]	5.48±0.11 [b]	5.03±0.12 [a]	5.06±0.21 [a]	*
Starch	64.86±1.63 [b]	58.37±2.23 [a]	62.07±1.82 [b]	57.54±3.62 [a]	***	64.15±3.50 [b]	61.05±2.37 [a]	65.36±2.05 [b]	59.31±1.36 [a]	**
Fiber										
NDF	13.18±2.65 [a]	13.31±1.38 [a]	12.67±2.48 [a]	17.50±0.92 [b]	**	13.75±2.31 [a]	14.21±1.86 [a]	12.61±1.44 [a]	16.81±0.63 [b]	**
ADF	2.68±0.27 [a]	2.55±0.28 [a]	3.47±0.73 [b]	3.25±0.32 [b]	**	2.54±0.22 [a]	2.60±0.36 [a]	3.59±0.24 [c]	3.09±0.41 [b]	***
ADL	0.29±0.21	0.29±0.10	0.37±0.20	0.31±0.16	ns	0.22±0.17 [a]	0.18±0.06 [a]	0.49±0.23 [b]	0.18±0.11 [a]	**
Cellulose	2.40±0.27 [a]	2.28±0.24 [a]	3.11±0.60 [b]	2.93±0.45 [b]	**	2.32±0.29 [a]	2.43±0.33 [a]	3.11±0.19 [b]	2.91±0.36 [b]	***
Total Ashes	2.34±0.20 [c]	2.09±0.05 [b]	1.81±0.08 [a]	2.10±0.11 [b]	***	1.91±0.05 [a]	2.34±0.06 [b]	1.98±0.29 [a]	1.97±0.04 [a]	***
Total Phenolics [(2)]	21.10±5.11 [a]	29.87±10.54 [b]	28.11±4.54 [b]	36.73±4.78 [c]	***	15.80±5.69 [a]	29.29±3.96 [b]	34.55±7.51 [c]	29.96±4.12 [b]	***
Gallic Acid [(3)]	4.30±1.52 [b]	2.76±1.66 [a]	5.74±2.77 [c]	3.98±1.49 [ab]	***	3.46±1.72	4.06±2.58	4.69±2.84	5.12±2.49	ns
Free Ellagic Acid [(3)]	4.84±1.37 [a]	8.99±1.03 [c]	7.14±1.48 [b]	7.45±0.97 [b]	***	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 desviation (SD) of mean.

[(1)] Data is presented as mean ± SD g/100g FW.

[(2)] Data is presented as mean ± SD mg gallic acid equivalents / gram fresh weight.

[(3)] Data is presented as mean ± SD mg phenolic / gram fresh weight.

SL - Significance Level.

ns - not significant (P>0.05); ns* - tendentiously significant (0.05<P<0.10); *, **, *** - significant at P<0.05, P<0.01 and P<0.001, respectively.

Within each row and for each one of the cultivars analysed, means with a different letter are significantly different.

Table 2.3 - Free amino acids contents (expressed as mg/100g FW, and DW in parentheses) of fresh (processing step A) raw shelled chestnut kernels from three cultivars[§].

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

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

Table 2.4 - Free amino acids Contents (expressed as mg/100g FW and DW in parentheses) of raw shelled chestnut kernels from two cultivars on the four processing steps analysed[§].

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

[§] T = trace (detected in some samples, but levels too low to be accurately quantified). Essential amino acids are highlighted in bold text. SL - Significance Level.

ns - not significant (P>0.05); *, **, *** - significant at P<0.05, P<0.01 and P<0.001, respectively. Within each row and for each one of the cultivars analysed, means with a different letter are significantly different.

2.4 Supporting Information

Table 2.5 - Effect of the factors of variation considered (cultivars and processing steps) and respective interaction on basic chemical composition (g/100g DM) and phenolics (mg/g FW) of raw shelled chestnut kernels[(§)].

	CULTIVARS				PROCESSING STEPS						INTERACTION	
	Judia	**Longal**	P	Var	**A**	**B**	**C**	**D**	P	Var	**Cult.*Proc. Step**	
Parameters				(%)						(%)	P	Var. (%)
Dry Matter [(1)]	51.37±2.44	50.95±2.72	0.431	0.0	52.12±3.33 [b]	51.94±2.93 [b]	51.20±1.51 [b]	49.37±1.01 [a]	0.003	0.0	0.000	30.9
Crude Fat	2.18±0.53	2.01±0.58	0.145	1.3	1.64±0.34 [a]	1.75±0.64 [a]	2.58±0.14 [b]	2.41±0.20 [b]	0.000	26.5	0.701	0.0
Crude Protein (N x 5.30)	5.10±0.27	5.17±0.30	0.327	0.0	5.00±0.39 [a]	5.35±0.21 [b]	5.08±0.17 [a]	5.13±0.23 [a]	0.010	3.6	0.084	5.2
Starch	60.71±3.77 [a]	62.47±3.35 [b]	0.017	3.4	64.50±2.63 [b]	59.71±2.60 [a]	63.71±2.52 [b]	58.42±2.77 [a]	0.000	23.9	0.217	1.6
Fiber												
NDF	14.16±2.72	14.34±2.22	0.737	0.0	13.46±2.39 [a]	13.76±1.63 [a]	12.64±1.93 [a]	17.15±0.83 [b]	0.000	22.0	0.727	0.0
ADF	2.99±0.57	2.96±0.53	0.768	0.0	2.61±0.24 [a]	2.58±0.31 [a]	3.53±0.52 [c]	3.17±0.36 [b]	0.000	26.2	0.750	0.0
ADL	0.31±0.16	0.26±0.20	0.312	0.0	0.25±0.18 [a]	0.23±0.09 [a]	0.43±0.21 [b]	0.24±0.15 [a]	0.014	4.9	0.255	1.6
Cellulose	2.68±0.53	2.69±0.44	0.902	0.0	2.36±0.27 [a]	2.35±0.29 [a]	3.11±0.42 [b]	2.92±0.39 [b]	0.000	22.1	0.894	0.0
Total Ashes	2.09±0.23	2.05±0.22	0.394	0.0	2.13±0.26 [bc]	2.22±0.14 [c]	1.89±0.22 [a]	2.04±0.11 [b]	0.000	0.0	0.000	36.9
Total Phenolics [(2)]	28.95±8.64	27.40±8.87	0.132	0.0	18.45±5.97 [a]	29.58±7.85 [b]	31.33±6.93 [bc]	33.34±5.58 [c]	0.000	17.6	0.000	7.7
Gallic Acid [(3)]	4.20±2.17	4.33±2.47	0.715	0.0	3.88±1.66 [ab]	3.41±2.24 [a]	5.21±2.82 [c]	4.55±2.10 [bc]	0.004	1.1	0.037	2.6
Free Ellagic Acid [(3)]	7.10±1.92	7.03±3.32	0.779	0.0	3.77±1.52 [a]	9.76±2.34 [c]	7.05±1.37 [b]	7.69±1.08 [b]	0.000	35.2	0.000	6.7

[(§)] Tabulated values are sample means ± standard deviation (SD) of mean.

[(1)] Data is presented as mean ± SD g/100g FW; [(2)] Data is presented as mean ± SD mg gallic acid equivalents / gram fresh weight; [(3)] Data is presented as mean ± SD mg phenolic / gram fresh weight.

Within each row and for each one of the factors considered, means with a different letter are significantly different.

Table 2.6 - Effect of the factors of variation considered (cultivars and processing steps) and respective interaction on free amino acids contents (expressed as mg/100g FW and DW in parentheses) of raw shelled chestnut kernels[§].

	CULTIVARS				PROCESSING STEPS						INTERACTION	
Parameters	**Judia**	**Longal**	P	Var (%)	**A**	**B**	**C**	**D**	P	Var (%)	**Cult.*Proc. Step** P	Var. (%)
Tyr	T	T			T	T	T	T				
Trp	T	T			T	T	T	T				
Phe	5.26 ± 3.49 (10.05 ± 7.14)	4.74 ± 1.04 (9.44 ± 2.03)	0.446 (0.391)	0.0 (0.0)	4.10 ± 1.07 [a] (7.49 ± 3.05) [a]	3.36 ± 2.18 [a] (6.68 ± 4.56) [a]	7.09 ± 2.95 [c] (14.31 ± 5.32) [c]	5.16 ± 1.77 [b] (10.49 ± 3.65) [b]	0.000 (0.000)	0.0 (0.0)	0.000 (0.000)	39.0 (58.8)
Asp	45.63 ± 23.56 [b] (85.83 ± 41.43) [b]	36.82 ± 18.46 [a] (74.20 ± 38.03) [a]	0.000 (0.005)	4.4 (2.2)	69.41 ± 17.30 [c] (131.17 ± 29.94) [c]	40.92 ± 11.28 [b] (78.39 ± 20.76) [b]	18.74 ± 5.18 [a] (36.43 ± 10.63) [a]	37.27 ± 9.24 [b] (75.68 ± 18.96) [b]	0.000 (0.000)	46.7 (46.8)	0.373 (0.547)	0.1 (0.0)
Glu	75.95 ± 29.07 [b] (145.61 ± 47.25)	67.60 ± 30.92 [a] (136.17 ± 63.97)	0.003 (0.031)	0.0 (0.0)	93.18 ± 27.07 [c] (175.40 ± 44.24) [c]	91.25 ± 23.07 [c] (176.59 ± 50.95) [c]	41.45 ± 13.68 [a] (80.60 ± 27.93) [a]	61.70 ± 19.23 [b] (129.96 ± 31.21) [b]	0.000 (0.000)	21.9 (19.9)	0.000 (0.000)	15.8 (19.8)
Asn	132.46 ± 40.58 [b] (254.83 ± 80.77) [b]	113.85 ± 32.33 [a] (226.75 ± 66.57) [a]	0.000 (0.003)	0.0 (0.0)	144.46 ± 29.47 [b] (278.43 ± 55.85) [b]	106.23 ± 28.18 [a] (205.89 ± 62.88) [b]	137.83 ± 43.26 [b] (266.63 ± 88.30) [b]	104.42 ± 30.33 [a] (212.40 ± 62.37) [a]	0.000 (0.000)	1.7 (0.0)	0.000 (0.000)	22.7 (31.1)
Gln	14.36 ± 3.78 [b] (27.55 ± 7.88) [b]	10.40 ± 3.49 [a] (20.83 ± 7.30) [a]	0.000 (0.000)	17.5 (10.4)	15.48 ± 2.71 [b] (29.57 ± 4.79) [b]	9.13 ± 1.62 [a] (17.43 ± 2.77) [a]	10.02 ± 4.15 [a] (19.41 ± 8.40) [a]	14.35 ± 3.64 [b] (29.19 ± 7.51) [b]	0.000 (0.000)	19.8 (17.8)	0.000 (0.000)	7.5 (15.7)
Arg	39.30 ± 11.26 (76.23 ± 24.22)	36.02 ± 12.20 (71.66 ± 24.65)	0.089 (0.307)	0.0 (0.0)	27.72 ± 9.60 [a] (52.89 ± 17.73) [a]	39.87 ± 12.07 [bc] (77.83 ± 27.11) [b]	43.78 ± 8.72 [c] (84.75 ± 18.16) [b]	36.85 ± 11.21 [b] (74.94 ± 23.04) [b]	0.000 (0.000)	0.0 (0.0)	0.000 (0.000)	28.4 (15.7)
Ser	6.63 ± 2.53 [b] (12.69 ± 5.01) [b]	5.28 ± 1.73 [a] (10.57 ± 3.53) [a]	0.000 (0.006)	0.0 (0.0)	6.24 ± 1.79 [b] (12.01 ± 3.17) [b]	5.34 ± 1.82 [ab] (10.33 ± 3.97) [ab]	7.55 ± 2.70 [c] (14.70 ± 5.47) [c]	4.48 ± 1.33 [a] (9.10 ± 2.71) [a]	0.000 (0.000)	0.0 (0.0)	0.000 (0.000)	27.7 (35.2)
Gly	T	T			T	T	T	T				
Thr	T	T			T	T	T	T				
Ala	22.33 ± 15.84 (43.28 ± 31.65)	21.30 ± 11.57 (42.27 ± 23.04)	0.471 (0.254)	0.0 (0.0)	22.90 ± 12.58 [b] (44.74 ± 26.80) [b]	12.68 ± 4.75 [a] (24.65 ± 10.20) [a]	37.94 ± 11.63 [c] (73.73 ± 23.78) [c]	12.28 ± 4.31 [a] (24.95 ± 8.81) [a]	0.000 (0.000)	18.2 (11.5)	0.000 (0.000)	44.5 (51.7)
Val	9.19 ± 3.27 (17.85 ± 7.01)	9.66 ± 4.44 (19.16 ± 8.30)	0.010 (0.003)	0.0 (0.0)	6.91 ± 1.29 [a] (13.29 ± 2.46) [a]	8.17 ± 3.94 [a] (16.03 ± 8.47) [a]	12.54 ± 4.34 [c] (24.21 ± 8.06) [c]	10.19 ± 2.89 [b] (20.72 ± 5.93) [b]	0.000 (0.000)	8.9 (8.0)	0.000 (0.000)	15.0 (17.1)
Ile	7.42 ± 2.78 (14.26 ± 5.62)	7.13 ± 3.55 (14.12 ± 6.81)	0.654 (0.393)	0.0 (0.2)	4.74 ± 1.14 [a] (9.12 ± 2.07) [a]	6.94 ± 2.86 [b] (13.27 ± 5.25) [b]	9.31 ± 2.53 [c] (17.98 ± 4.72) [c]	8.69 ± 3.68 [c] (17.63 ± 7.44) [c]	0.000 (0.000)	13.9 (15.5)	0.482 (0.857)	0.0 (0.0)
Leu	5.69 ± 2.55 [b] (11.25 ± 5.25) [b]	3.24 ± 1.76 [a] (6.37 ± 3.26) [a]	0.000 (0.000)	12.4 (12.5)	1.82 ± 0.59 [a] (3.48 ± 1.02) [a]	2.87 ± 0.72 [b] (5.83 ± 1.46) [b]	6.68 ± 1.62 [d] (12.93 ± 3.29) [d]	4.80 ± 2.08 [c] (9.76 ± 4.27) [c]	0.000 (0.000)	36.1 (34.1)	0.000 (0.000)	9.3 (11.7)
Total	363.8 (698.4)	316.0 (631.5)			397.0 (757.6)	327.3 (633.5)	332.9 (645.7)	300.2 (614.8)				

[§] T = trace (detected in some samples. but levels too low to be accurately quantified). Essential amino acids are highlighted in bold text.

Within each row and for each one of the factors considered. means with a different letter are significantly different.

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CHAPTER 3

This is the first two year study with the major aim of evaluating the effects at the four sequential major stages of industrial processing (A-fresh, B-after storage during 3 months at ±0°C and RH=90%, C-after industrial peeling by flame or fire at 800 to 1000 ºC, D-after freezing in a tunnel with a CO_2 flow at -65 °C) on the contents of important nutrients in the fruits of four Portuguese (Judia, Longal, Martaínha and Lada) and two European ("Spanish" - Puga do Bolo, and "Italian" - Viterbes) cultivars of *Castanea sativa* Mill. Fruits were found to contain significant contents of crude energy but low fat. In both harvest years, the interaction cultivar x processing step had the greatest influence on the starch contents while the cultivar factor had a much greater influence on the dry matter content. The fibre contents were more influenced by the processing stage for both harvests years.

Keywords: *Castanea sativa*; industrial processing; starch; fat; energy; fibre.

Industrial Processing Effects on Chestnut Fruits (*Castanea sativa* Mill.) 1. Starch, Fat, Energy and Fibre

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3.1 Introduction

Chestnut fruit is an important economic food resource in Portugal, especially in the Northeast, as well as in many other European countries. This is due to the favourable climatic, edaphic and ecological conditions that this area provides. In the Portuguese fruit exportation sector, the chestnut is one of the major ones. In the Northeast it accounts for 86% of the total fruit exported (Ferreira-Cardoso, 2002). Not only is chestnut an important economic resource but also the chestnut fruit has been subject of intensive research in the last decade, mainly because of the potential human health benefits that the intake of this nut might provide. There have been a few studies on the composition of various nutrients and bioactives in the raw fruits (stage A in the processing scheme), but no analyses of the whole industrial process have previously been done.

There are a few basic chemical studies of chestnut fruits evaluating the contents of moisture, fibre, crude protein, crude fat, ashes, minerals, amino acids and phenolics (Pereira-Lorenzo *et al*., 2006; De Vasconcelos *et al*., 2007). Starch content has been shown to be high in Spanish and Italian chestnut cultivars, and additionally studies have been performed on Italian chestnuts to evaluate the specific contents of amylose and amylopectin (Attanasio *et al*., 2004; Pereira-Lorenzo *et al*., 2006; Pizzoferrato *et al*., 1999). Fruits of Portuguese cultivars have low crude fat content which is low in saturated fatty acids and high in unsaturated fatty acids (Ferreira-Cardoso, 2002). A few analyses of the crude energy in raw chestnut kernels have been done and they reveal significant values showing that the chestnuts can be used as an important source of dietary energy (Desmaison, & Adrian, 1986).

The purpose of this two year study was, for the first time to determine the effects of a sequential four-stage industrial processing (fresh, stored, peeled and frozen) on the contents of starch, fat, energy and fibre in the fruits of four different Portuguese and two selected European cultivars of *Castanea sativa* Mill. from two harvests. It is important to identify cultivars with higher levels of nutrients and other biologically-active compounds that have positive effects on human health as part of the promotion of this plant food. It is also important to assess any

changes that occur in during industrial processing of the fruits, so that processing can be optimized for composition and quality.

3.2 Materials and Methods

3.2.1 Plant Material and Processing

The Portuguese cultivars analyzed in the first year (2006) were Longal, Judia and Martaínha, and the two other cultivars were Spanish ("Puga do Bolo" from Galiza region) and Italian ("Viterbes"/"Marron Vero" from Viterbo region). Only Portuguese cultivars were analyzed in the second year (2007); Longal, Judia, Martaínha and Lada. The data presented in this paper is from years 2 and 3 (2006 and 2007, respectively) of a three year project, VALCAST II. The project focused on evaluating fruit components in the chestnut cultivars through the four stages of industrial processing. In both years the cultivars were harvested in October/November from orchards where they are mainly implanted. The samples were collected at Sortegel-Produtos Congelados S.A. (Bragança - Portugal), a chestnut processing/commercialization company; fresh (19%) and frozen (74%) fruits. The samples were collected at the end of each processing step: (A) fresh; (B) after storage during 3 months at ±0 °C and RH=90%; (C) after industrial peeling by flame or fire ("brûlage") at high temperatures (800 to 1000 ºC) during 1 to 2 seconds in a rotary cylindrical oven; and (D) after freezing in a tunnel with a CO_2 flow at -65 °C during 15 to 20 minutes (Figure 3.1). For each cultivar, at each of the four processing stages, three samples were collected. The samples from stages A and B were stored in a refrigerator at ±2 °C, for a maximum of 3 days until they were hand-peeled. Samples from stage C were processed immediately, and samples from stage D were kept at -20 °C until analyzed. For the samples from stages A and B the shells and pellicle were manually removed. The raw shelled kernel samples were broken into small pieces approximately 1 cm^2, 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 48 h), to determine dry matter and for use in the other analyses (Ferreira-Cardoso *et al*., 1999). The 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).

3.2.2 Chemicals

All basic chemicals and reagents were of analytical grade and were obtained from a commercial source (Reagente 5, Porto - Portugal; Sigma/Aldrich, Merck, and Pronalab).

3.2.3 Determination of Dry Matter, Organic Matter and Ash Contents

Air-dried samples (2.5g) in duplicate were analyzed for residual water content and total ash content using previously validated methods (De Vasconcelos *et al*., 2007). In the first step, samples were dried 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, USA) for 3 h at 550 °C, and the ash content was obtained.

3.2.4 Extraction and Quantification of Starch

Starch was extracted and analyzed as previously described (De Vasconcelos *et al*., 2007). Essentially, sub-samples of the air-dried samples (50mg) in triplicate for each sample were analyzed. The method involves the conversion of starch to glucose during two stages of an enzymatic treatment (α-amylase followed by amyloglucosidase) then colorimetric determination of the glucose released using a glucose-specific coupled enzyme reaction in combination with a 4-amino antipyrine chromogen system.

3.2.5 Crude Fat Extraction and Measurements

Sub-samples of the air-dried samples (3g), 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 (AOAC, 1990).

3.2.6 Crude Energy Measurements

Sub-samples of the air-dried material were weighed in duplicate (2 x 1g) and the crude energy (caloric value) was obtained through the total calorific energy released in the combustion of the sample in a calorimeter PARR mod. 6300.

3.2.7 Extraction and Quantification of Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF), and Acid Detergent Lignin (ADL)

Sub-samples were weighed (1.0g for Neutral Detergent Fibre and 0.8g for Acid Detergent Fibre and Acid Detergent Lignin) in duplicate, and the respective quantifications of NDF, ADF, and ADL were determined by the Van Soest detergent system (Robertson, & Van Soest, 1981). Because of the high starch content of the samples, α-amylase (a thermo resistant enzyme from Sigma Chemical Co., ref. A-4582) was also used to enzymatically transform the chains of amylose and amylopectin to make them more susceptible to extraction from the fibre fraction.

3.2.8 Statistical Analysis

Data was analyzed by two-way analysis of variance (ANOVA) followed by Duncan's new multiple range test with a 0.05 significance level, using the *SuperANOVA* (1.1 version, Abacus Concepts Inc., 1989) statistical package. Cultivars and processing steps were the sources of variation considered, having as error term the fixed effect of these factors. The percent contribution of each one of the sources (cultivar, processing step and their interaction cultivar x processing step) for the total variation observed was calculated as described in the papers of Snedecor & Cochran (1980) and Sokal & Rohlf (1981).

3.3 Results and Discussion

3.3.1 Presentation of Tabulated Data

The data from these studies is presented in the following way; in both tables under the heading "Cultivar" the data presented is the mean of all processing stages (A to D) for each specific cultivar shown. Under the heading "Processing Stage" each individual stage (A to D) is the mean of all the cultivars (shown under the "Cultivar" heading) for that year for that specific stage.

3.3.2 Dry Matter Contents

The present study contained high contents of dry matter from both harvest years (2006 and 2007) and these contents varied significantly for each of the processing stages (Table 3.1). In 2006 the highest dry matter content was found in Martaínha (50.4g $100g^{-1}$ fresh weight), while in 2007 (Table 3.2) the highest value was found in the cultivar Judia (58.6g $100g^{-1}$ fresh weight)

compared with the other cultivars of the same year or with the same cultivar in the previous year. The dry matter contents found in the cultivars that are common to both years were significantly higher in 2007, in Longal but especially in Judia and with less significance in Martaínha. With the exception of the cultivars Martaínha and "Spanish" in 2006, and Lada in 2007, all the other cultivars presented a variation of the dry matter contents and therefore in the content of water as a result of the dehydration (during storage) followed by re-hydration (during the washing and cleaning after mechanical peeling) and further dehydration (freezing). An increase of the dry matter contents (decrease of water) was observed from stage A to B, followed by a decrease in the samples of stages C and D. The high moisture content in the chestnut kernels could allow enzymatic reactions to continue during the first weeks after harvest causing some chemical changes (mainly in sugars and vitamins) and is often responsible for the growth of fungi (Desmaison, & Adrian, 1986). The two-way analysis of variance (Tables 3.1 and 3.2), show that the cultivar factor had more of an effect on the variation of the dry matter content for both harvests, with more statistical significance in 2007 harvest (71%) (Table 3.2). The contribution either of the processing stage or the interaction between these two factors was more significant in 2006 (14% and 12%, respectively). The values of dry matter found in the present study in stage A are within the ranges of previous studies for chestnuts from Spain (between 40.3 and 60.1g $100g^{-1}$ fresh weight) and Portugal (between 46.3 and 53.3g $100g^{-1}$ fresh weight) (Pereira-Lorenzo *et al*.2006; Borges *et al*., 2008). The values of dry matter found in the present study for 2006 harvest are lower than the values presented in samples from a previous work (De Vasconcelos *et al*., 2007), but the values for 2007 harvest show higher dry matter contents, especially for the cultivars Judia and Longal. This fact may be related with an intense influence of the climatic factors in these years.

3.3.3 Total Ashes Contents

In 2006 (Table 3.1) the highest values of total ash contents were found in the "Italian" cultivar (2.7g $100g^{-1}$ DM) and the lowest in Martaínha (2.0g $100g^{-1}$ DM). In 2007 (Table 3.2) the highest values of total ashes were found in Lada and Judia cultivars (2.5 and 2.6g $100g^{-1}$ DM, respectively) and the lowest again in Martaínha (2.3g $100g^{-1}$ DM). Possibly due to the difference in the nature of the cultivars used and the resultant behaviour during the processing stages in 2006, the ash contents decreased from stage A (2.3% DM) to B (2.2% DM) and then to stage C (2.0% DM), increasing in stage D (2.3% DM), in 2007 an increase from stage A to B was visible (2.2 and 2.6% DM, respectively) and a decrease in stage D (2.4% DM). The factors account for

the variation in 2006 were the cultivar (27%) and mainly the interaction cultivar x processing stage (62%), with no contribution from the processing stage factor alone (Table 3.1). In 2007 (Table 3.2), the factors account for the variation were the cultivar (25% DM) and the processing stage (27% DM). The ash content for the Portuguese cultivars in 2006 and 2007 were consistent with the previous analyses (De Vasconcelos *et al*., 2007). The total ash content was previously reported in chestnut fruits from Spain (between 1.8 and 3.2g $100g^{-1}$ DM) and Portugal (between 1.5 and 2.2g $100g^{-1}$ DM) (Pereira-Lorenzo *et al*., 2006; Borges *et al*., 2008).

3.3.4 Total Starch Contents

In 2006 the content of starch (Table 3.1) shows significant changes only between the foreign cultivars, while in 2007, the significant changes were found in Lada and mainly in Longal (Table 3.2). In addition a decrease of the starch content from stage A to B was observed in both harvests, but the decrease being more significant in 2006. A decrease in the starch content was seen after the storage period (three months at ±0 ºC) especially in the "Italian" and Longal cultivars, with the exception being Judia in both years and of Martaínha in 2007. The starch content variation was more influenced by the interaction cultivar x processing stage than by either factor alone for both 2006 and 2007 (14% and 32%, respectively). Although the factors cultivar and processing stage had less influence in the starch content, their influence was greater in 2006 than in 2007. The Portuguese cultivars contained higher contents of starch, with the lowest value being found in the "Italian" cultivar (48.7g $100g^{-1}$ DM) and the highest in Lada (54.5g $100g^{-1}$ DM), among all cultivars the highest values were found in stage A in 2006 (53.8g $100g^{-1}$ DM) and in 2007 (54.9g $100g^{-1}$ DM) (Tables 3.1 and 3.2). The values of starch presented in this study in stage A are within the range of a previous study in Spanish chestnut (between 42.2 and 66.6g $100g^{-1}$ DM) but higher than another in Portuguese chestnuts (38.6 and 47.9g $100g^{-1}$ DM) (Pereira-Lorenzo *et al*., 2006; Borges *et al*., 2008). The present results show that the values found in 2006 and 2007 are consistent, but both are lower than the data from the study of De Vasconcelos *et al*. (2007).

3.3.5 Crude Fat Contents

In the present study the average crude fat contents, considering all the samples, was of 2.6% DM (2006) and 2.7% DM (2007), with the cultivar Longal revealing the lowest value (1.9% DM) and the cultivar "Italian" the highest (4.4% DM) in the first harvest year. In 2007 the cultivar Lada showed the lowest content (2.0% DM) and Judia the highest (3.6% DM) (Tables

3.1 and 3.2). Therefore, the cultivar "Italian" is the richer in crude fat, while Longal and Martaínha in 2006 and Lada in 2007, had the lowest crude fat values. For the cultivars analyzed in both years, there was a substantial increase in crude fat contents from the first to the second year and although the average values show little variation, in absolute terms there was a significant variation in 2006 (C.V. = 38%) and in 2007 (C.V. = 23.5%). Based on the results of the two-way analysis of variance (Table 3.1) the cultivar was the factor that caused greater variation in the crude fat contents (71% in 2006 and 69% in 2007), while the effect of the processing stage factor was not consistent (4% in 2006 and 2% in 2007). The cultivars richest in crude fat are the "Italian" and Judia in 2006. The crude fat of the Portuguese cultivars in 2006 were consistent with the previous work of De Vasconcelos *et al*. (2007), but the 2007 samples contained the highest crude fat content. The present values of crude fat for processing stage A are close to the upper limit of the range presented by Borges *et al*. (2008) (1.7 to 3.1% DM) and Pereira-Lorenzo *et al*. (2006) (1.7 to 4.0% DM). Previous studies show high values of fat contents in walnuts, hazelnuts and almonds (64.2, 60.2 and 56.7 % DM, respectively) (Kornsteiner *et al*., 2006); these fat contents are much higher than the ones found in the present work for chestnuts.

3.3.6 Crude Energy Values

The current study shows that the average values of crude energy were between 376 and 432kcal $100g^{-1}$ DM in 2006 and between 406 and 439kcal $100g^{-1}$ DM in 2007, with a tendency to increase from stage A to B (Table 3.1), that might be explained by the increase in dry matter and therefore a decrease in moisture. The variation observed in the crude energy values was more affected by the processing stage (15% in 2006 and 36% in 2007) (Tables 3.1 and 3.2), and other influencing factors such as cultivar and the interaction cultivar x processing stage appeared to be less significant (9.7 and 1.2% in 2006 and 9.6% and 2.3% in 2007, respectively). The results show that the crude energy is strongly dependent on the crude fat contents, since the cultivars richer in fat ("Italian" and Judia) contained the highest caloric values (424.6 and 420.1-426.9kcal $100g^{-1}$ DM, respectively). The values of crude energy obtained in a previous study (Desmaison, & Adrian, 1986) were lower (370kcal $100g^{-1}$ DM) than the values obtained in the present study for processing stage A. A previous study of crude energy in hazelnuts showed a mean value of 657 kcal $100g^{-1}$ DM (Alasalvar *et al*., 2003).

3.3.7 Fibre Contents

3.3.7.1 NDF

In 2006 there was a clear increase in the NDF contents from stage A to D, with more statistical significance from A to B and from C to D (Table 3.1). In this year, the highest contents of NDF were found in the cultivars "Spanish" (19.9g $100g^{-1}$ DM) and Longal (17.5g $100g^{-1}$ DM) and the lowest contents in the "Italian" cultivar (13.8g $100g^{-1}$ DM) (Table 3.1).

3.3.7.2 ADF

In 2006 the highest value of ADF was found in the "Italian" cultivar (3.6g $100g^{-1}$ DM) and the lowest in the Judia cultivar (3.0 g $100g^{-1}$ DM). The processing stages revealed a significant increase from A to C (Table 3.1).

3.3.7.3 ADL

The values of ADL found in this study reveal no statistically significant differences between the cultivars or processing stages (Tables 3.1 and 3.2).

3.3.7.4 Cellulose

The "Italian" cultivar contained the highest content of cellulose (3.1g $100g^{-1}$ DM) in the 2006 harvest, while the Martaínha cultivar had the lowest content (2.6g $100g^{-1}$ DM). In this year the values of cellulose show a significant increase between the processing stages B to C (Table 3.1).

In 2007 the fibre constituents NDF, ADF and cellulose were not significantly different between the cultivars, but there was a more significant increase from stages B to C (Table 3.2). While in 2006 the variation in the contents of NDF, ADF and cellulose was due to the influence of the factors cultivar and especially the processing stage, in 2007 the factor cultivar had no contribution to this variation. These values reveal that the content of NDF found in 2006 are consistent with previous data from 2005 for the same Portuguese cultivars (De Vasconcelos *et al*., 2007), but the values in 2007 samples show higher NDF content. The same pattern can be seen with the ADF contents of 2007 samples. On the other hand the values of ADL found in 2006 were higher than the previous values found (De Vasconcelos *et al*., 2007), but lower than the values for 2007. The content of NDF and ADF have been previously analysed in Spanish

(values between 9.4 and 28.5g $100g^{-1}$ DM for NDF and 2.3 and 4.5g $100g^{-1}$ DM for ADF) and Portuguese chestnut kernels (13.8 and 24.4g $100g^{-1}$ DM for NDF and 1.9 and 3.2g $100g^{-1}$ DM for ADF) (Pereira-Lorenzo *et al*., 2006; Borges *et al*., 2008). A previous study shows the content of NDF and ADF in walnuts, with values between 3.3 and 4.4g $100g^{-1}$ DM and 2.2 and 3.8g $100g^{-1}$ DM, respectively (Savage, 2001). The chestnut kernels present a higher content of NDF than the walnuts, revealing once again that the chestnut kernels are a good source of fibre.

3.4 Conclusions

In both of the harvest years, regarding the basic composition, only for the crude energy did the processing stage factor have more of an effect on the total variation. On the other hand, the fat contents were significantly more affected by the cultivar factor than by the other two factors. The values of crude energy reveal a directly proportional relationship with the crude fat contents, because the cultivars with the highest fat contents had the highest energy values. The factor cultivar had the greatest influence on starch while fibre variation was more influenced by processing stage factor. The "Spanish" cultivar showed a significant decrease in the studied compounds during industrial processing. The decrease in starch during the storage period may be explained by the enzymatic catabolism of starch into soluble sugars. Overall the industrial processing had a positive effect (increase of values from stage A to D) on the crude energy and fibre contents (NDF, ADF and cellulose) of both harvest years, as well on the ash content of 2007. On the other hand the industrial processing had a negative effect on the starch and to a lesser extent on the fat contents. It is clear that further studies are required to select the best cultivars in terms of health-positive components.

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Table 3.1 - Effect of cultivar, processing step and respective interaction on dry matter content (g $100g^{-1}$ FW) and starch, crude fat, crude energy, fibre and total ashes levels (g $100g^{-1}$ DM) of raw shelled chestnut kernel samples (edible portion) of 2006 harvest [(1)].

	CULTIVAR							PROCESSING STAGE						INTERACTION	
	Judia	**Longal**	**Martaínha**	**"Italian"**	**"Spanish"**	P	Var (%)	**A**	**B**	**C**	**D**	P	Var (%)	**C*PS** P	**C*PS** Var.(%)
Dry Matter (g $100g^{-1}$FW)	47.86±1.21 ^{c}	46.95±1.56 ^{b}	50.36±0.65 ^{d}	46.94±0.94 ^{b}	46.48±0.62 ^{a}	0.000	53.3	47.02±1.79 ^{a}	48.76±1.71 ^{c}	47.06±1.59 ^{a}	48.04±1.34 ^{b}	0.000	13.7	0.000	11.5
Starch	52.12±2.66 ^{bc}	53.89±3.68 ^{c}	51.48±3.00 ^{b}	48.74±4.70 ^{ab}	50.12±2.23 ^{b}	0.000	7.4	53.82±3.97 ^{c}	48.80±3.19 ^{a}	51.19±2.22 ^{b}	51.26±3.65 ^{b}	0.000	9.6	0.002	14.2
Crude Fat	2.52±0.34 ^{b}	1.91±0.41 ^{a}	2.00±0.25 ^{a}	4.39±0.61 ^{c}	2.38±0.48 ^{b}	0.000	70.8	2.95±1.24 ^{b}	2.82±1.04 ^{b}	2.34±0.74 ^{a}	2.45±0.91 ^{a}	0.000	4.3	0.000	6.9
Crude Energy[(2)]	420.06±7.07 ^{cd}	412.77±12.24 ^{a}	414.58±7.78 ^{ab}	424.59±3.79 ^{d}	417.91±4.56 ^{bc}	0.000	9.7	410.09±12.78 ^{a}	419.17±4.78 ^{b}	419.83±3.05 ^{b}	422.84±3.70 ^{b}	0.000	14.7	0.323	1.2
Fibre															
NDF	16.74±2.91 ^{b}	17.47±2.60 ^{b}	16.93±3.18 ^{b}	13.76±3.07 ^{a}	19.94±4.21 ^{c}	0.000	18.3	12.68±1.99 ^{a}	17.50±3.28 ^{b}	17.78±2.14 ^{b}	19.92±2.93 ^{c}	0.000	36.8	0.113	2.4
ADF	3.01±0.41 ^{a}	3.07±0.47 ^{a}	3.07±0.43 ^{a}	3.59±0.65 ^{b}	3.50±0.61 ^{b}	0.000	7.1	2.78±0.30 ^{a}	3.15±0.54 ^{b}	3.46±0.44 ^{c}	3.62±0.53 ^{c}	0.000	17.3	0.038	6.4
ADL	0.36±0.14 ^{a}	0.44±0.25 ^{a}	0.47±0.29 ^{a}	0.45±0.24 ^{a}	0.42±0.29 ^{a}	0.865	0.0	0.35±0.21 ^{a}	0.47±0.26 ^{a}	0.44±0.22 ^{a}	0.45±0.27 ^{a}	0.603	0.0	0.754	0.0
Cellulose	2.65±0.45 ^{a}	2.63±0.63 ^{a}	2.60±0.45 ^{a}	3.14±0.55 ^{b}	3.07±0.59 ^{b}	0.002	4.8	2.40±0.40 ^{a}	2.68±0.60 ^{a}	3.02±0.49 ^{b}	3.17±0.49 ^{b}	0.000	11.3	0.041	7.0
Total Ashes	2.06±0.21 ^{b}	2.10±0.21 ^{bc}	1.96±0.10 ^{a}	2.68±0.42 ^{d}	2.15±0.45 ^{c}	0.000	26.8	2.27±0.23 ^{c}	2.18±0.33 ^{b}	2.01±0.18 ^{a}	2.31±0.63 ^{c}	0.000	0.0	0.000	62.4

[(1)] Tabulated values are sample means ± standard deviation (SD) of mean. [(2)] Data are presented as means ± SD kcal/100g dry matter.

NDF - Neutral Detergent Fibre (corresponds to total cell wall constituents, i. e. hemicelluloses, cellulose and lignin); ADF - Acid Detergent Fibre (corresponds to cellulose more lignin contents); ADL - Acid Detergent Lignin (corresponds to lignin content); C - Cultivar; PS - Processing stage.

Within each row and for each one of the factors considered, means with a different letter are significantly different (P<0.05), according to Duncan New Multiple Range Test.

Table 3.2 - Effect of cultivar, processing step and respective interaction on Dry Matter content (g $100g^{-1}$ FW) and Starch, Crude Fat, Crude Energy, Fibre and Total Ashes levels (g $100g^{-1}$ DM) of raw shelled chestnut kernel samples (edible portion) of 2007 harvest [(1)].

	CULTIVAR						PROCESSING STAGE						INTERACTION	
	Judia	**Longal**	**Martaínha**	**Lada**	P	Var (%)	**A**	**B**	**C**	**D**	P	Var (%)	**C*PS** P	**C*PS** Var. (%)
Dry Matter (g $100g^{-1}$ FW)	58.62±2.56 ^{d}	55.07±2.67 ^{c}	49.80±2.45 ^{b}	46.22±1.22 ^{a}	0.000	71.0	51.81±3.85 ^{ab}	54.62±6.62 ^{c}	52.32±5.29 ^{b}	50.96±5.05 ^{a}	0.000	4.1	0.000	5.5
Starch	52.42±1.72 ^{a}	51.65±4.80 ^{a}	52.16±1.83 ^{a}	54.54±2.66 ^{b}	0.002	0.0	54.88±3.67 ^{b}	52.38±1.79 ^{a}	52.54±2.92 ^{a}	50.98±2.83 ^{a}	0.000	3.7	0.000	31.7
Crude Fat	3.56±0.47 ^{c}	2.74±0.29 ^{b}	2.63±0.25 ^{b}	2.00±0.22 ^{a}	0.000	68.5	2.61±0.33 ^{a}	2.97±0.65 ^{c}	2.78±0.78 ^{b}	2.56±0.71 ^{a}	0.000	1.9	0.000	16.1
Crude Energy[(2)]	426.92±8.75 ^{c}	424.72±6.52 ^{bc}	423.44±5.55 ^{b}	418.81±5.76 ^{a}	0.000	9.6	414.27±4.04 ^{a}	427.84±5.41 ^{b}	426.29±5.84 ^{b}	425.49±3.91 ^{b}	0.000	35.6	0.179	2.3
Fibre														
NDF	17.32±2.71 ^{a}	17.24±2.42 ^{a}	17.92±2.52 ^{a}	17.24±2.42 ^{a}	0.599	0.0	16.29±2.14 ^{a}	16.47±2.03 ^{a}	19.39±1.50 ^{b}	18.24±2.06 ^{b}	0.000	10.5	0.004	15.7
ADF	3.49±0.60 ^{ab}	3.58±0.54 ^{ab}	3.64±0.50 ^{b}	3.36±0.54 ^{a}	0.135	0.0	3.05±0.39 ^{a}	3.34±0.42 ^{b}	4.13±0.40 ^{c}	3.54±0.28 ^{b}	0.000	29.3	0.007	11.1
ADL	0.49±0.25 ^{a}	0.57±0.25 ^{a}	0.59±0.26 ^{a}	0.48±0.26 ^{a}	0.630	0.0	0.50±0.23 ^{a}	0.46±0.29 ^{a}	0.58±0.23 ^{a}	0.59±0.27 ^{a}	0.510	0.0	0.375	1.0
Cellulose	3.00±0.56 ^{a}	3.02±0.69 ^{a}	3.05±0.56 ^{a}	2.88±0.59 ^{a}	0.785	0.0	2.55±0.39 ^{a}	2.89±0.50 ^{ab}	3.55±0.49 ^{c}	2.95±0.51 ^{b}	0.000	14.4	0.063	7.0
Total Ashes	2.58±0.24 ^{c}	2.34±0.12 ^{b}	2.25±0.18 ^{a}	2.53±0.19 ^{c}	0.000	24.7	2.20±0.06 ^{a}	2.55±0.12 ^{c}	2.54±0.27 ^{c}	2.40±0.21 ^{b}	0.000	26.5	0.003	8.6

[(1)] Tabulated values are sample means ± standard deviation (SD) of mean. [(2)] Data are presented as means ± SD kcal/100g dry matter.

NDF - Neutral Detergent Fibre (corresponds to total cell wall constituents, i. e. hemicelluloses, cellulose and lignin); ADF - Acid Detergent Fibre (corresponds to cellulose more lignin contents); ADL - Acid Detergent Lignin (corresponds to lignin content); C - Cultivar; PS - Processing stage.

Within each row and for each one of the factors considered, means with a different letter are significantly different ($P<0.05$), according to Duncan New Multiple Range Test.

3.5 Supporting Information

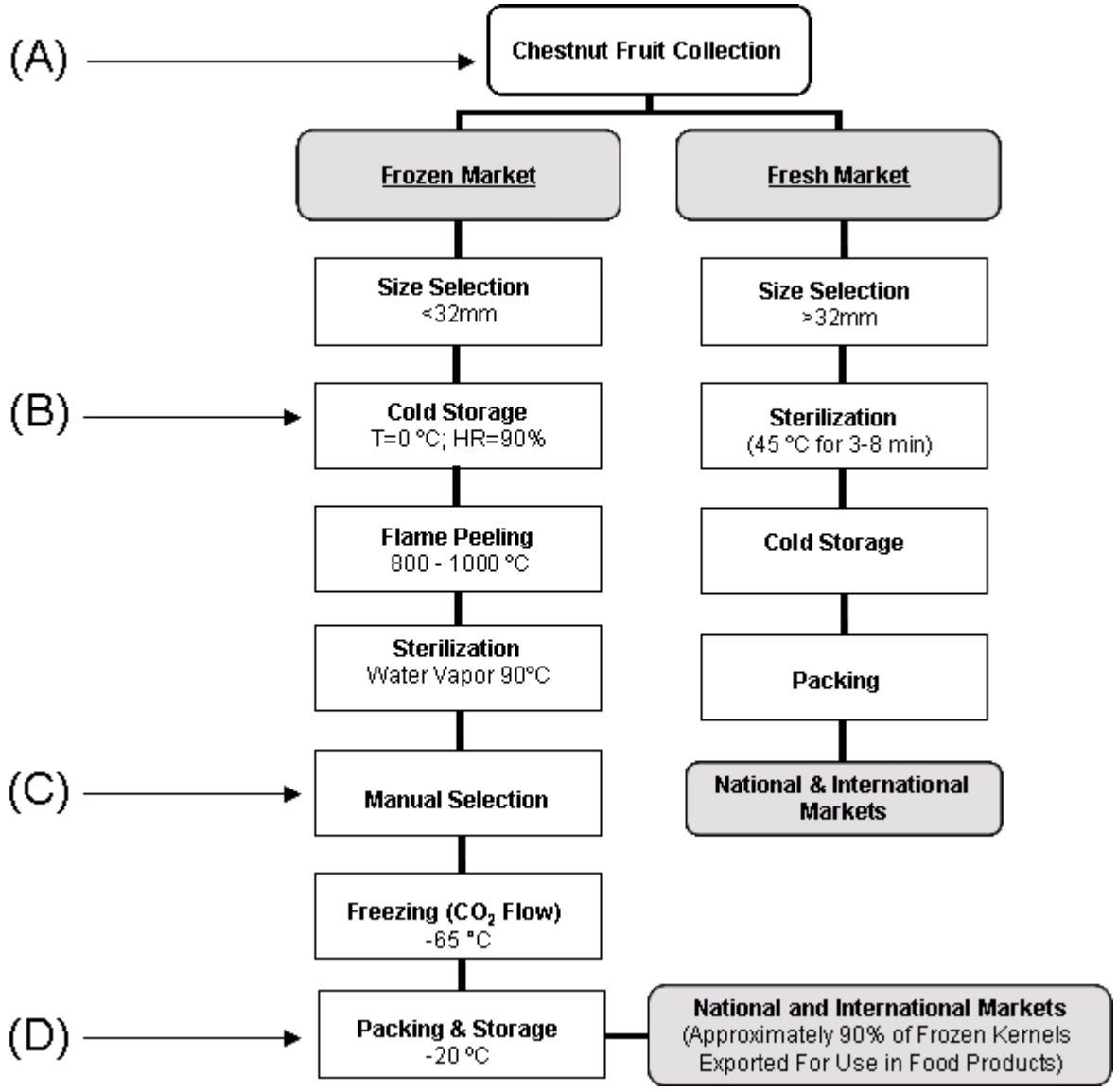

Figure 3.1 - Flow chart showing the major industrial processing stages for chestnut fruits (Samples were taken at the indicated stages A, B, C and D).

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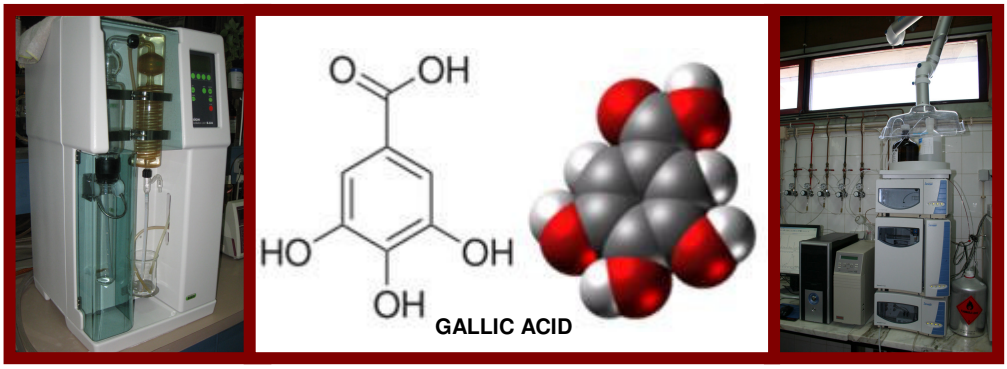
CHAPTER 4



The aim of this study was to analyse the effects of industrial processing on the composition of proteins, free amino acids and phenolics in *Castanea sativa* Mill. fruits of two years. The Portuguese cultivars were Judia, Longal, Martaínha and Lada, and the European cultivars were "Spanish" (Puga do Bolo) and "Italian" (Viterbes). At all processing stages the fruits contained low contents of protein (4.1-5.4g $100g^{-1}$ DM). The essential amino acid Thr had the highest values (3.6-10.0mg $100g^{-1}$ FW), while the highest non-essential amino acid was Asn (14.9-36.4mg $100g^{-1}$ FW). Total phenolics (6.6-18.3mg g^{-1} FW) were more significantly affected by the processing stage factor. In 2007 the gallic acid contents were more affected by the processing stage and interaction factors (cultivar x processing stage), whereas the cultivar factor had more of an effect on ellagic acid. Industrial processing has some negative effects, specifically reducing the contents of some amino acids.

Keywords: *Castanea sativa*; industrial processing; protein; free amino acids; phenolics; gallic acid; ellagic acid.

Industrial Processing Effects on Chestnut Fruits (*Castanea sativa* Mill.) 2. Crude Protein, Free Amino Acids and Phenolic Phytochemicals

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4.1 Introduction

The increasing concern about food and health effects has promoted more studies on the chestnut fruit, focused not only on the chemical composition but also on the positive benefits to human health. Part of these studies has included evaluation of the major nutrients (crude fat, fatty acids, crude protein, total and free amino acids, and mono-, di- and poly-saccharides) and minerals in the raw materials. There are only a few papers on non-nutrient bioactives (such as polyphenols) of chestnut fruits, and very few studies have reported on the effects of industrial processing on the composition of chestnut fruits.

The amino acids, in free form or derived from the metabolism of dietary proteins are of high importance. Amino acids are biologically active, possessing multiple biological functions as sources of energy and as precursors of proteins and other important molecules. Amino acids are divided into two classes: essential (the human body does not synthesize them in enough quantities, and they are necessary in the diet) and non-essential (they can be synthesized in adequate concentrations in the body). Total amino acids (including free and protein-derived), from acid hydrolyses studies of chestnut fruits, have been analyzed in Portuguese (*Castanea sativa*) (Borges *et al*., 2008), American (*Castanea dentata*), Italian (*Castanea sativa*) and Chinese (*Castanea mollisima*) chestnuts (Meredith *et al*., 1988). The common predominant protein-derived amino acids were aspartic acid and glutamic acid (Desmaison *et al*., 1984). Plant tissues also contain free amino acids such as in *Castanea sativa* fruits. The predominant free amino acids in another previous work were identified as aspartic acid, asparagine and glutamic acid (De Vasconcelos *et al*., 2007).

The presence of phenolics (gallic and ellagic acid) in the chestnut fruits has also been reported (De Vasconcelos *et al*., 2007). Both of these acids have been associated with various positive health effects including antioxidant effects, decreases in the risk of cardiovascular diseases, anticancer mechanisms and anti-inflammatory properties (Veluri *et al*., 2006; Mertens-Talcott *et al*., 2006; Hooper *et al*., 2008).

The purpose of the second part of this two year study was to evaluate in detail, for the first time, the changes in crude protein, free amino acids and phenolic phytochemicals during four-stage industrial processing (fresh, stored, peeled and frozen chestnuts) of the fruits of four different Portuguese and two other selected European cultivars of *Castanea sativa* Mill.

4.2 Materials and Methods

4.2.1 Plant Samples and Processing

The Portuguese cultivars analyzed in the first year (2006) were Longal, Judia and Martaínha, and the two other cultivars were Spanish ("Puga do Bolo" from Galiza region) and Italian ("Viterbes"/"Marron Vero" from Viterbo region). Only Portuguese cultivars were analyzed in the second year (2007); Longal, Judia, Martaínha and Lada. In both years the cultivars were harvested in October/November from orchards where they are mainly implanted. The samples were collected at Sortegel-Produtos Congelados S.A. (Bragança - Portugal), an enterprise involved in processing chestnut and commercialization of this fruit. The samples were collected at the end of each processing step: (A) fresh; (B) after storage during 3 months at ±0 °C and HR=90%; (C) after industrial peeling by flame or fire ("brûlage") at high temperatures (800 to 1000 ºC) during 1 to 2 seconds in a rotary cylindrical oven; and (D) after freezing in a tunnel with a CO_2 flow at -65 °C during 15 to 20 minutes (for details see Figure 4.1 and the first part of this work, De Vasconcelos *et al*., 2009). For each one of the cultivars at each of the four processing stages, three samples of 1.5kg each were collected. The samples from stages A and B were stored in a refrigerator at ±2 °C, for a maximum of 3 days until they were hand-peeled. 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. For the samples from stages A and B the shells and pellicle were manually removed. The raw shelled kernel samples were broken into small pieces approximately 1 cm^2, 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 48 h), to determine dry matter and for use in crude protein analyses. Another portion was frozen with liquid nitrogen, powdered, and freeze-dried in a Dura Dry μP from F.T.S. Systems (Stone Ridge, New York) for at least 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), which was also used for

powering the freeze-dried samples. The reason for processing the samples using two different drying methods were that for protein and free amino acids air-drying is the recommended standard protocol. To ensure negligible changes in free amino acids and phenolics, such as formation of artefacts, it is necessary to freeze-dry the samples thus during extraction there are no enzymatic reactions leading analyte degradation or the formation of protein-phenolic complexes. All of the extraction protocols are standardized and previously validated.

4.2.2 Chemicals

All chemicals and reagents were of analytical grade and were obtained from a commercial source (Reagente 5, Porto, Portugal; Sigma/Aldrich, USA; Merck/VWR, Carnaxide, Portugal; Pronalab, Brazil). All solvents were of high-performance liquid chromatography (HPLC) grade, and all water was ultrapure. All amino acids and phenolic (gallic acid, ellagic acid, and naringin) standards were obtained from a commercial source (Reagente 5, Porto - Portugal).

4.2.3 Crude Protein Extraction and Measurements

The total nitrogen content of sub-samples of the air-dried samples (1g in duplicate) was analyzed using the Kjeldahl method in combination with a selenium catalyst through a Büchi 435 digestion unit and a Büchi B-324 (Flawil, Switzerland) distillation unit (AOAC, 1990). The crude protein content was calculated by using 5.3 as the conversion factor (CP = Total N x 5.3), accordingly to McCarthy and Meredith (1988).

4.2.4 Free Amino Acids Extraction and Analysis

The freeze-dried samples were weighed in triplicate (0.2 g) into 15 mL glass centrifuge tubes. Extraction was performed following the method previously described by De Vasconcelos *et al*. (2007). A mixture of amino acid standards was freshly prepared and run in parallel.

4.2.5 Extraction of Phenolics for Total Phenolics and HPLC Analyses

Essentially triplicate samples (3 x 40mg; A, B and C) of each of the freeze-dried chestnut samples were extracted in 2mL screw-cap micro-tubes with 70% v/v methanol at 70^{o}C for 30 min, with vortex mixing for 10 seconds (LABINCO L46 at maximum speed) every 5 min to optimise the extraction; replicates A and B had 1000µL of 70% v/v methanol added, replicate C had 950µL of 70% v/v methanol and 50µL 1mg/mL naringin (internal standard) added (De

Vasconcelos *et al*., 2007). The total phenolics were determined using the Folin-Ciocalteau method previously described (Javanmardi *et al*., 2003).

4.2.6 Statistical Analysis

Data were analyzed by two-way analysis of variance (ANOVA) followed by Duncan's new multiple range test with a 0.05 significance level, using the *SuperANOVA* (1.1 version, Abacus Concepts Inc., 1989) statistical package. Cultivars and processing steps were the sources of variation considered, having as error term the fixed effect of these factors. The percent contribution of each one of the sources (cultivar, processing stage and their interaction cultivar x processing stage) for the total variation observed was calculated as described by Snedecor and Cochran (1980) and Sokal and Rohlf (1981).

4.3 Results and Discussion

4.3.1 Crude Protein

With the exception of the Martaínha cultivar in both harvest years and Lada cultivar in 2007, there were significant variations in the crude proteins of other cultivars during the processing stages. Considering all the processing stages and both harvest years, significant differences between the cultivars were also detected. The cultivar factor contributed to the observed crude protein content variations with 56% (2006) and 36% (2007), while the contribution of the processing stage factor had only some significance (11%) in 2007 (Tables 4.1 and 4.2). In 2006 the highest contents of crude protein were found in the cultivars Judia and Longal, considering all the processing stages, with average values of 5.4 and 5.3% DM, respectively, while the cultivar "Italian" had the lowest value (4.1% DM). In 2007 the lowest crude protein content was found in Judia (4.9% DM), probably due to the larger size/caliber of this cultivar (dilution effect), while the highest content was found in Lada (5.8% DM). Considering all the cultivars, the lower values in 2006 were found in stages A and D (4.9 and 4.8% DM, respectively) and in 2007 in stage A (4.9% DM) (Tables 4.1 and 4.2). Similar to the results from most of nuts and oilseeds, the crude protein contents obtained in our studies reveal a high negative correlation between the contents of crude fat and crude protein ($r = -0.648$ in 2006 and $r = -0.691$ in 2007; $P<0.001$) (De Vasconcelos *et al.* 2009). This fact indicates that the formation of protein and fat during the maturation process of the chestnut fruit is in this case is inversely related (co-occurrence of high protein and low fat contents or vice versa). The crude

protein contents of the Portuguese cultivars from the 2006 harvest were relatively higher than those reported in a previous study (De Vasconcelos *et al*., 2007) but lower than those of the 2007 harvest samples. The values of crude protein presented in this study for processing stage A were close to the lower limit values obtained by Borges *et al*. (2008) (between 4.9 and 7.4g $100g^{-1}$ DM) and Pereira-Lorenzo *et al*. (2006) (between 4.5 and 9.6g $100g^{-1}$ DM) for Portuguese and Spanish fresh chestnut cultivars, respectively. Previous analyses determined the protein content in hazelnuts (15.97g $100g^{-1}$ DM) and almonds (between 16.07 and 31.46g $100g^{-1}$ DM) were higher than those of chestnut fruits (Alasalvar *et al*., 2003; Askin *et al*., 2007). The recommended daily intake of proteins and amino acids for adults (56g for males and 46g for females) was previously reported (USDA, 2009).

4.3.2 Free Amino Acids

In the present study, 15 amino acids were found in the fruits of the chestnut cultivars studied: Asp, Glu, Asn, Ser, Gln, Gly, Thr, Arg, Ala, Tyr, Val, Trp, Ile, Leu, and Phe. Gly was only present in trace amounts (Tables 4.1 and 4.2). Among the 15 amino acids seven are essential (Arg, Ile, Leu, Thr, Val, Phe, and Trp). Considering all the processing stages, the essential amino acid found with the highest content in the 2006 harvest year was Thr in the "Spanish" cultivar (9.99g $100mg^{-1}$ FW), while the non-essential amino acid occurred at the highest level was Asn in Judia (36.38g $100mg^{-1}$ FW). When considering all the cultivars, the processing stage was the factor that contributed less to the variation observed of the essential amino acid Thr (12.2%), but was the highest contribution for the non-essential amino acids Asp (25.6%) and Ala (28.7%) (Table 4.1). The essential amino acids values were affected more significantly by the cultivar factor and especially the interaction between the two factors. The non-essential amino acids Ala and Asp were, however, more affected by the processing. A decrease in the free amino acid contents from stage B to D may be associated with the roles of these amino acids in the biosynthesis of phenolics such as tannins. For the 2007 samples, the essential amino acid at the highest level was Thr in Longal (9.28mg $100g^{-1}$ FW), while the lower were Ile, Leu and Phe in Judia (0.31, 0.34 and 0.46mg $100g^{-1}$ FW, respectively) (Table 4.2). The cultivar factor had a significant effect on the contents of the essential amino acids Val, Phe and Ile (58.4, 66.6 and 65.0%, respectively), while the contents of Thr, Arg and Leu were more significantly affected by the processing stage factor (32.9, 37.1 and 41.6%, respectively). The non-essential amino acid Asn was more significantly affected by the cultivar factor (46.0%), while Asp, Glu, Ser, Ala and Tyr were more affected by the processing stage factor (59.4, 40.2,

45.0, 70.7 and 62.5%, respectively). The non-essential amino acid with the highest values was Asn, with Tyr having the lowest content (Table 4.2). The decreases observed in the last stages of processing, for some of the amino acids, may be explained by lower thermo-stabilities during stage C (heat peeling). The values found in this study are much lower than the values presented in a previous work (De Vasconcelos *et al*., 2007), however, the amino acids that were below the detection limits in those previous analyses, show detectable levels in the current work. Previous studies analyzed the free amino acid contents in hazelnuts showing the highest values in Arg (204.92mg $100g^{-1}$ FW) and Glu (128.68mg $100g^{-1}$ FW), while the essential amino acids with the highest values were Thr (19.73mg $100g^{-1}$ FW) and Val (19.36mg $100g^{-1}$ FW) (Alasalvar *et al*., 2003).

4.3.3 Total Phenolics

In the present study the total phenolics values from 2006, considering all the cultivars, show an increase from stage A to D. The cultivars that had the highest contents of total phenolics were Martaínha and the "Spanish" cultivar (11.6 and 11.5mg g^{-1} FW, respectively) (Table 4.3). The general increase in the content of total phenolics may be explained by their synthesis via the shikimic acid pathway. This pathway is involved in the biosynthesis of hydrolyzable tannins (gallo- and ellagi-tannins) and aromatic amino acids that are precursors for many classes of phenolics. Previous works describe the formation of gallic acid directly derived from an early intermediate of the shikimate pathway; 5-dehydroshikimate (Werner *et al*., 2004). In 2007, considering all the processing stages, the highest total phenolic contents were found in the cultivar Longal (18.3mg g^{-1} FW), and when considering all the cultivars there was a significant increase from stage A to B (Table 4.3). Total phenolics were significantly affected by the processing stage factor in both 2006 and 2007 (18.6 and 32.3%, respectively). The total phenolics content in chestnut fruits was previously analyzed in Portuguese cultivars during industrial processing (between 21.10 and 36.73mg g^{-1} FW) (De Vasconcelos *et al*., 2007). In the present study the values, for the fruits of processing stage A, of total phenolics were lower than previously found which may be related to climatic and nutritional factors. The total phenolics content of almonds (without skin) (0.47mg g^{-1} FW), hazelnuts (2.91mg g^{-1} FW) and walnuts (16.25mg g^{-1} FW) were previously analyzed revealing lower values than those found in chestnut fruits (Kornsteiner *et al*., 2006).

4.3.4 Free Gallic and Ellagic Acids

In 2006, considering all the processing stages, the highest contents of free gallic acid was found in the cultivars Judia and "Italian" (24.9 and 22.7mg g^{-1} FW, respectively), while the "Spanish" and Martaínha cultivars had the highest contents of free ellagic acid (47.8 and 45.1mg g^{-1} FW, respectively) (Table 4.3). Considering all the cultivars, there was an increase from stage A to B followed by a decrease from B to C, and this was especially significant for the free ellagic acid contents (Table 4.3). The factors cultivar, processing stage and the interaction were evenly responsible for the variations observed. In 2007, considering all the processing stages, the cultivar Martaínha had the highest contents of free gallic acid (13.1mg g^{-1} FW) and free ellagic acid (36.8mg g^{-1} FW). Considering all the cultivars, there was an increase in these phenolics from stage A to C, and was more significant in absolute values for ellagic acid (Table 4.3). This increase in ellagic acid may be explained by the hydrolysis of endogenous ellagitannins, releasing HHDP (3,4,5,3´,4´,5´-hexahydroxydiphenic acid) which spontaneously rearranges to form ellagic acid (Gross, 2008). The variation in gallic acid contents was more affected by the factors processing stage (29.2%) and interaction (26.5%), while the factor cultivar had more of an effect on the free ellagic acid contents (31.9%). The content in gallic acid (between 2.76 and 9.07mg g^{-1} FW) and free ellagic acid (between 2.71 and 10.52mg g^{-1} FW) was previously analyzed in Portuguese cultivars during industrial processing (De Vasconcelos *et al*., 2007). In the present study the gallic acid contents were within the range previously presented. The values of ellagic acid from 2007 harvest were also close to the previous values, but the ellagic acid values in 2006 harvest were higher than those previously found (De Vasconcelos *et al*., 2007). Previous studies analyzed the free ellagic acid content in walnuts revealing a range between 0.09 and 0.32mg g^{-1} FW (Li *et al*., 2006), while the gallic acid contents in hazelnut kernels was 1.27mg g^{-1} FW (Shahidi *et al*., 2007). These results show that the chestnut kernels are richer in free gallic and ellagic acids that are known to have positive antioxidant effects.

4.4 Conclusions

The crude protein contents were significantly more affected by the cultivar factor than by the other two factors. The total phenolics content was more significantly affected by processing stage, increasing from stage A to D, and may be related to the biosynthesis/turnover of phenolics during the processing stages. Ellagic acid significantly increased from stage A to B in both harvest years which may be explained by the formation of ellagic acid from ellagitannin catabolism, as previously discussed. Almost all the amino acids increased from stage A to B,

most significantly for Thr. This increase was followed by a general decrease to stage C, which might be explained by the occurrence of Maillard reactions (Morini and Maga, 1995). Overall the industrial processing had a positive effect (increase of values from stage A to D) on the total phenolics, gallic and ellagic acid contents of both harvest years, and on the crude protein of 2007. In the 2006 harvest year there were positive effects on the essential amino acids Thr and Leu, and on the non-essential amino acids Ser, Ala, and Tyr. In the same harvest year the non-essential amino acids Asp and Glu presented a significant decrease between stages A and D. In the 2007 harvest, positive effects due to the industrial processing were visible in the majority of the amino acids found.

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Table 4.1 - Effect of cultivar, processing step and respective interaction on crude protein (g $100g^{-1}$ DM) and amino acids content (mg $100g^{-1}$ FW) of raw shelled chestnut kernel samples (edible portion) of 2006 harvest [(1)]

	CULTIVAR							PROCESSING STAGE						INTERACTION	
	Judia	**Longal**	**Martaínha**	**"Italian"**	**"Spanish"**	P	Var (%)	**A**	**B**	**C**	**D**	P	Var (%)	**C*PS** P	**C*PS** Var. (%)
Crude Protein (Ntotal x 5.3)	5.40±0.20 ^{d}	5.28±0.54 ^{c}	4.82±0.11 ^{b}	4.13±0.11 ^{a}	5.20±0.45 ^{c}	0.000	55.7	4.89±0.62 ^{a}	5.12±0.64 ^{c}	5.02±0.52 ^{b}	4.83±0.47 ^{a}	0.000	0.0	0.000	31.6
Asp	7.91±6.30 ^{b}	7.83±4.79 ^{b}	5.23±4.52 ^{a}	7.77±4.25 ^{b}	5.67±3.58 ^{ab}	0.035	2.2	10.08±3.90 ^{c}	9.36±5.27 ^{c}	2.28±0.80 ^{a}	5.64±3.00 ^{b}	0.000	25.6	0.000	17.0
Glu	14.06±6.66 ^{b}	11.58±5.00 ^{ab}	9.43±6.22 ^{a}	11.39±5.29 ^{ab}	8.41±5.48 ^{a}	0.010	4.1	14.72±6.21 ^{b}	12.90±6.98 ^{b}	6.96±2.42 ^{a}	9.15±3.51 ^{a}	0.000	12.3	0.000	16.1
Asn	36.38±12.83 ^{c}	27.05±11.59 ^{b}	21.26±4.19 ^{ab}	17.53±6.12 ^{a}	26.27±9.93 ^{b}	0.000	14.7	24.00±14.07 ^{a}	30.94±13.39 ^{b}	25.02±6.88 ^{a}	22.84±7.94 ^{a}	0.021	3.0	0.002	14.0
Ser	2.03±0.61 ^{b}	2.40±1.19 ^{bc}	2.67±0.76 ^{c}	1.44±0.29 ^{a}	3.39±1.56 ^{d}	0.000	16.8	1.61±0.70 ^{a}	3.22±1.70 ^{c}	2.44±0.60 ^{b}	2.28±0.71 ^{b}	0.000	13.9	0.002	11.2
Gln	2.71±0.69 ^{b}	3.29±1.05 ^{c}	2.23±0.85 ^{a}	2.75±0.43 ^{b}	3.22±0.93 ^{c}	0.000	8.6	2.67±1.12 ^{a}	3.33±0.94 ^{b}	2.84±0.63 ^{a}	2.51±0.58 ^{a}	0.001	5.5	0.000	23.6
Gly	T	T	T	T	T			T	T	T	T				
Thr	8.10±2.53 ^{b}	8.13±4.86 ^{b}	6.85±2.33 ^{b}	3.80±1.25 ^{a}	9.99±3.81 ^{c}	0.000	16.7	4.94±3.07 ^{a}	9.54±4.92 ^{c}	8.18±2.58 ^{bc}	6.84±2.41 ^{b}	0.000	12.2	0.000	18.0
Arg	1.28±0.44 ^{b}	1.22±0.58 ^{b}	1.18±1.02 ^{b}	0.53±0.50 ^{a}	1.81±0.77 ^{c}	0.000	12.3	0.96±0.78 ^{a}	1.53±1.14 ^{b}	1.23±0.25 ^{ab}	1.09±0.68 ^{a}	0.026	2.7	0.000	20.3
Ala	6.94±3.24 ^{b}	6.42±3.45 ^{b}	7.68±4.37 ^{b}	4.67±2.44 ^{a}	6.70±2.67 ^{b}	0.010	3.6	3.26±0.61 ^{a}	6.25±3.75 ^{b}	9.97±1.54 ^{c}	6.47±2.49 ^{b}	0.000	28.7	0.025	6.6
Tyr	0.71±0.33 ^{a}	0.76±0.35 ^{a}	0.81±0.59 ^{a}	0.72±0.23 ^{a}	1.05±0.26 ^{b}	0.014	3.7	0.63±0.42 ^{a}	0.89±0.44 ^{b}	0.90±0.27 ^{b}	0.83±0.35 ^{b}	0.022	3.0	0.000	25.0
Val	1.92±0.43 ^{b}	2.33±0.91 ^{bc}	2.34±0.86 ^{bc}	1.38±0.44 ^{a}	2.68±0.78 ^{c}	0.000	12.6	1.91±0.72 ^{a}	2.52±1.12 ^{b}	2.20±0.60 ^{ab}	1.89±0.66 ^{a}	0.010	3.9	0.004	12.3
Trp	0.68±0.59 ^{a}	0.39±0.45 ^{a}	0.53±0.53 ^{a}	0.66±0.85 ^{a}	0.66±0.88 ^{a}	0.519	0.0	0.74±0.79 ^{b}	0.74±0.85 ^{b}	0.34±0.43 ^{a}	0.51±0.50 ^{ab}	0.158	1.0	0.000	28.8
Phe	1.51±0.37 ^{b}	1.58±0.46 ^{b}	1.68±0.64 ^{b}	1.16±0.42 ^{a}	1.81±0.42 ^{b}	0.006	5.8	1.34±0.31 ^{a}	1.76±0.66 ^{b}	1.65±0.34 ^{ab}	1.44±0.56 ^{ab}	0.035	3.1	0.081	5.7
Ile	1.01±0.25 ^{ab}	1.19±0.45 ^{bc}	1.27±0.55 ^{bc}	0.81±0.18 ^{a}	1.42±0.30 ^{c}	0.000	9.8	0.99±0.41 ^{a}	1.28±0.57 ^{b}	1.21±0.35 ^{ab}	1.10±0.27 ^{ab}	0.056	2.3	0.010	10.9
Leu	0.86±0.41 ^{ab}	0.93±0.48 ^{b}	1.01±0.83 ^{b}	0.57±0.25 ^{a}	0.94±0.29 ^{b}	0.068	2.3	0.60±0.34 ^{a}	0.94±0.72 ^{b}	0.97±0.31 ^{b}	0.94±0.45 ^{b}	0.039	2.8	0.002	15.4

[(1)] Tabulated values are sample means ± standard deviation of mean (SD). Essential amino acids are highlighted in bold text.

T- Trace amounts (detected in some samples but with levels too low to be accurately quantified). C - Cultivar; PS - Processing stage.

Within each row and for each one of the factors considered, means with a different letter are significantly different (P<0.05), according to Duncan New Multiple Range Test.

Table 4.2 - Effect of cultivar, processing step and respective interaction on crude protein (g $100g^{-1}$ DM) and amino acids content (mg $100g^{-1}$ FW) of raw shelled chestnut kernel samples (edible portion) of 2007 harvest [(1)]

	CULTIVAR						PROCESSING STAGE						INTERACTION	
	Judia	**Longal**	**Martaínha**	**Lada**	P	Var (%)	**A**	**B**	**C**	**D**	P	Var (%)	**C*PS** P	**C*PS** Var. (%)
Crude Protein (Ntotal x 5.3)	4.87±0.27 [a]	5.19±0.50 [b]	5.17±0.21 [b]	5.77±0.18 [c]	0.000	35.5	4.91±0.56 [a]	5.38±0.37 [b]	5.39±0.23 [b]	5.31±0.42 [b]	0.000	10.9	0.004	9.3
Asp	5.42±2.94 [a]	8.45±4.04 [b]	9.41±4.15 [c]	8.90±3.16 [bc]	0.000	14.0	8.63±1.57 [b]	9.49±3.17 [c]	2.81±1.53 [a]	11.25±1.99 [d]	0.000	59.4	0.000	8.0
Glu	10.29±4.90 [ab]	11.77±5.54 [bc]	12.49±5.58 [c]	9.12±2.96 [a]	0.005	3.2	13.89±3.30 [c]	13.67±5.22 [c]	4.58±0.75 [a]	10.87±1.51 [b]	0.000	40.2	0.000	20.5
Asn	14.88±3.33 [a]	26.63±5.55 [b]	31.59±5.25 [c]	24.21±3.56 [b]	0.000	46.0	22.08±7.08 [a]	25.95±10.71 [b]	23.96±5.47 [ab]	25.33±6.21 [b]	0.027	2.0	0.000	13.5
Ser	3.03±1.23 [ab]	3.41±1.57 [b]	2.94±0.87 [ab]	2.80±0.98 [a]	0.080	1.5	1.38±0.27 [a]	3.47±1.15 [b]	3.84±0.49 [b]	3.48±0.43 [b]	0.000	45.0	0.039	5.3
Gln	5.29±2.03 [b]	5.97±4.65 [b]	3.43±1.01 [a]	3.58±0.78 [a]	0.001	7.6	2.59±0.53 [a]	7.32±4.16 [c]	3.98±1.02 [b]	4.39±1.03 [b]	0.000	20.9	0.015	9.4
Gly	T	T	T	T			T	T	T	T				
Thr	4.50±1.84 [a]	9.28±4.80 [c]	7.94±2.30 [b]	8.13±2.26 [bc]	0.000	19.2	3.58±1.57 [a]	9.62±4.27 [c]	8.72±1.57 [bc]	7.93±2.03 [b]	0.000	32.9	0.000	11.7
Arg	0.86±0.55 [a]	1.16±0.40 [b]	1.34±0.36 [c]	1.34±0.42 [c]	0.000	12.1	0.77±0.21 [a]	1.14±0.54 [b]	1.70±0.19 [c]	1.10±0.28 [b]	0.000	37.1	0.000	16.1
Ala	6.19±2.07 [a]	7.58±2.62 [b]	6.20±2.42 [a]	7.33±2.86 [b]	0.000	5.3	3.97±0.63 [a]	7.55±1.65 [c]	10.04±1.27 [d]	5.74±0.53 [b]	0.000	70.7	0.000	7.9
Tyr	0.58±0.30 [a]	0.60±0.20 [a]	0.56±0.15 [a]	0.79±0.26 [b]	0.000	12.1	0.43±0.07 [a]	0.58±0.13 [b]	0.98±0.17 [c]	0.55±0.14 [b]	0.000	62.5	0.000	7.7
Val	0.79±0.38 [a]	1.32±0.33 [b]	1.59±0.44 [c]	2.60±0.50 [d]	0.000	58.4	1.32±0.51 [a]	1.56±0.89 [b]	1.96±0.77 [c]	1.45±0.82 [ab]	0.000	7.4	0.000	8.4
Trp	1.31±0.61 [a]	1.73±0.71 [b]	1.99±0.56 [b]	1.12±0.66 [a]	0.000	12.3	1.33±0.92 [a]	2.19±0.72 [b]	1.37±0.20 [a]	1.26±0.33 [a]	0.000	15.1	0.005	12.2
Phe	0.46±0.20 [a]	0.68±0.19 [b]	0.82±0.26 [c]	1.72±0.34 [d]	0.000	66.6	0.77±0.40 [a]	0.81±0.54 [a]	1.19±0.59 [b]	0.90±0.58 [a]	0.000	7.2	0.005	4.3
Ile	0.31±0.17 [a]	0.59±0.12 [b]	0.72±0.18 [c]	1.34±0.27 [d]	0.000	65.0	0.65±0.27 [a]	0.73±0.49 [a]	0.89±0.46 [b]	0.69±0.46 [a]	0.000	3.4	0.003	5.6
Leu	0.34±0.26 [a]	0.43±0.19 [b]	0.49±0.17 [c]	0.77±0.26 [d]	0.000	30.1	0.27±0.11 [a]	0.48±0.26 [b]	0.80±0.19 [c]	0.47±0.20 [b]	0.000	41.6	0.000	7.1

[(1)] Tabulated values are sample means ± standard deviation of mean (SD). Essential amino acids are highlighted in bold text.

T- Trace amounts (detected in some samples but with levels too low to be accurately quantified). C - Cultivar; PS - Processing stage.

Within each row and for each one of the factors considered, means with a different letter are significantly different (P<0.05), according to Duncan New Multiple Range Test.

Table 4.3 - Effect of cultivar, processing step and respective interaction on total and specific phenolics content of raw shelled chestnut kernel samples (edible portion) of 2006 and 2007 harvests.

	CULTIVAR							PROCESSING STAGE						INTERACTION	
	2006														
	Judia	**Longal**	**Martaínha**	**"Italian"**	**"Spanish"**	P	Var (%)	**A**	**B**	**C**	**D**	P	Var (%)	**C*PS** P	**C*PS** Var. (%)
Total Phenolics[(1)]	7.87±4.44 [a]	7.66±3.60 [a]	11.60±2.85 [b]	9.16±3.69 [a]	11.48±1.57 [b]	0.000	10.0	6.57±3.69 [a]	9.02±3.71 [b]	10.89±1.81 [c]	12.02±2.73 [c]	0.000	18.6	0.000	14.6
Gallic Acid[(2)]	24.89±23.08 [c]	9.92±5.68 [a]	14.91±7.44 [b]	22.71±11.51 [c]	10.26±3.80 [a]	0.000	27.4	7.47±3.12 [a]	19.68±13.07 [c]	14.32±4.05 [b]	25.61±20.08 [d]	0.000	29.9	0.000	32.9
Ellagic Acid[(2)]	19.32±10.15 [a]	16.05±10.34 [a]	45.07±17.03 [c]	26.66±8.28 [b]	47.78±36.33 [c]	0.000	29.5	13.36±8.84 [a]	51.29±31.53 [d]	27.14±11.97 [b]	33.00±17.91 [c]	0.000	31.0	0.000	26.7
	2007														
	Judia	**Longal**	**Martaínha**	**Lada**		P	Var (%)	**A**	**B**	**C**	**D**	P	Var (%)	**C*PS** P	**C*PS** Var. (%)
Total Phenolics[(1)]	13.49±3.09 [a]	18.30±5.25 [b]	16.93±2.95 [b]	13.44±3.31 [a]		0.000	17.4	10.57±2.04 [a]	17.52±4.97 [b]	17.15±3.03 [b]	16.92±1.59 [b]	0.000	32.3	0.002	10.0
Gallic Acid[(2)]	8.03±1.79 [a]	10.00±3.82 [b]	13.09±7.60 [c]	8.58±1.83 [ab]		0.000	16.8	6.05±1.42 [a]	11.64±4.13 [c]	13.11±5.48 [d]	8.82±1.96 [b]	0.000	29.2	0.000	26.5
Ellagic Acid[(2)]	7.28±4.98 [a]	33.48±22.75 [b]	36.78±24.16 [b]	7.53±4.73 [a]		0.000	31.9	3.83±2.83 [a]	27.78±22.07 [b]	32.60±25.18 [b]	24.77±18.69 [b]	0.000	19.0	0.000	16.6

[(1)] Data are presented as means ± SD mg gallic acid equivalents/gram fresh weight. [(2)] Data are presented as means ± SD mg phenolic/gram fresh weight.

Within each row and for each one of the factors considered, means with a different letter are significantly different (P<0.05), according to Duncan New Multiple Range Test.

C - Cultivar; PS - Processing stage.

4.5 Supporting Information

For each one of the cultivars at each of the four processing stages, three samples of 1.5kg each were collected. The samples from stages A and B were stored in a refrigerator at ±2 °C, for a maximum of 3 days until they were hand-peeled. 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. For the samples from stages A and B the shells and pellicle were manually removed. The raw shelled kernel samples were broken into small pieces approximately 1 cm^2, 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 48 h), to determine dry matter and for use in crude protein analyses. Another portion was frozen with liquid nitrogen, powdered, and freeze-dried in a Dura Dry μP from F.T.S. Systems (Stone Ridge, New York) for at least 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), which was also used for powering the freeze-dried samples. The reason for processing the samples using two different drying methods were that for protein and free amino acids air-drying is the recommended standard protocol. To ensure negligible changes in free amino acids and phenolics, such as formation of artefacts, it is necessary to freeze-dry the samples thus during extraction there are no enzymatic reactions leading analyte degradation or the formation of protein-phenolic complexes. All of the extraction protocols are standardized and previously validated.

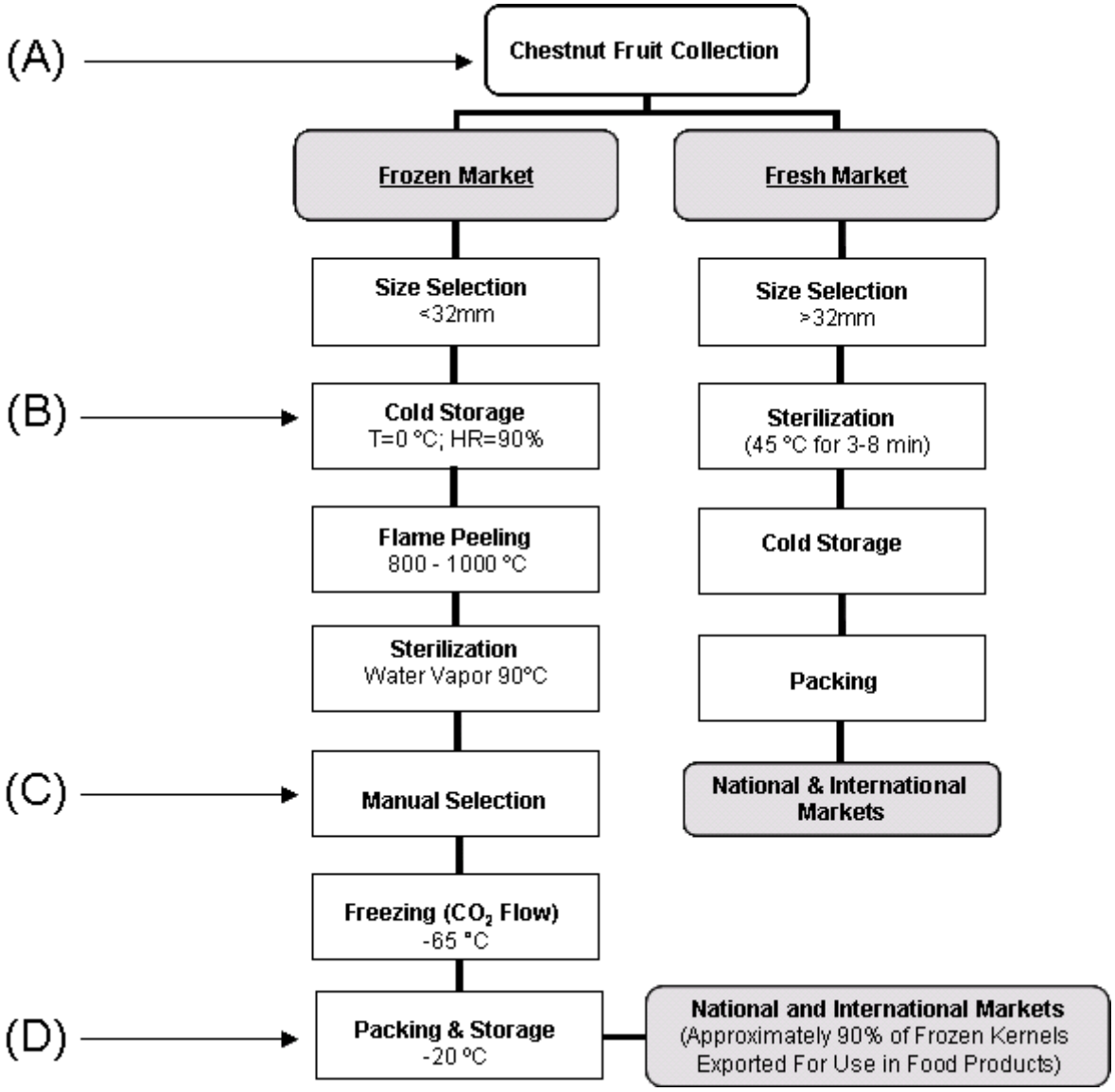

Figure 4.1 - SORTEGEL processing scheme with sampling stages (A, B, C and D) indicated.

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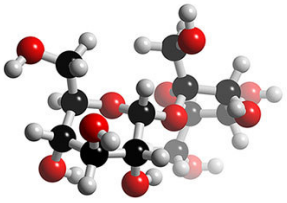
CHAPTER 5



SUCROSE

The major aim of this study was to evaluate the effects of industrial processing on the composition of minerals, free sugars, vitamins and pigments in fruits of chestnut cultivars at four stages of industrial processing (A-fresh; B-after 3 months storage at ±0°C and relative humidity = 90%; C-after flame peeling at 800-1000 ºC for 1-2 seconds; D-after freezing in a tunnel with a CO_2 flow at -65 °C for 15-20 minutes) from two harvest years. Potassium and phosphorous were predominant in the fruits of all cultivars for both harvest years. Calcium, magnesium, iron, zinc and manganese were also present in the fruits. Fruits from both harvest years had a significant content of free sugars, with sucrose predominating, and these sugars were more affected by the processing stage. Significant levels of lutein, lutein esters, γ-tocopherol and vitamin C were also found in the chestnut fruits. Fruit carotenoids and vitamin C significantly decreased during the industrial processing.

Keywords: *Castanea sativa*; industrial processing; minerals; free sugars; vitamin C; vitamin E; carotenoids.

Industrial Processing Effects on Chestnut Fruits (*Castanea sativa* Mill.) 3. Minerals, Free Sugars, Carotenoids and Antioxidant Vitamins

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5.1 Introduction

The availability of more types of transformed chestnut fruit products has lead to a higher consumer demand and consequently increased economic and cultural importance, especially in the Northern and Central regions of Portugal leading to increased export of chestnuts and some chestnut products (mainly frozen chestnut fruits) (Ferreira-Cardoso *et al*., 1993; Ferreira-Cardoso, 2002). The production of these nuts in Portugal was of 30,886t in 2006 (INE, 2008). The fresh fruits can be sold directly to the consumers and used mainly in gastronomy (traditional recipes), exported fresh and frozen, and they can also be sold as "transformed" fruits, a market almost nonexistent in Portugal, but well developed in other countries like Spain, France, Switzerland and Italy ("marron glacés", tinned or sugar-coated chestnuts, in liquors and also purees, chestnut creams, flours, soups or yogurts). There is still considerable data lacking for the contents of minerals, vitamins and pigments in chestnut fruits and very few reports on the effects of industrial processing on the composition.

Previous analyses of mineral contents in raw chestnut fruits indicate the presence of important macro-elements (Ca, P, K, Mg and S) with potassium representing the majority of this group and also important micro-elements (Fe, Cu, Zn and Mn) (Ferreira-Cardoso, 2002; Pereira-Lorenzo *et al*., 2006; Borges *et al*., 2008). Chestnut fruits are rich in carbohydrates with starch predominating and significant levels of the free sugar sucrose (saccharose). Previous studies identified mono- and di-saccharides (glucose, fructose, sucrose and maltose) in chestnut fruits. These sugars are very important for the assessment of the chestnut fruit commercial quality (Bernárdez *et al*., 2004; Attanasio *et al*., 2004).

There is only one previous study on the content of carotenoids in chestnut fruits and only beta-carotene (pro-vitamin A), lutein and zeaxanthin were detected (USDA, 2008). Vitamins are essential in a balanced diet for various health related effects. Humans cannot synthesize vitamin C (ascorbic acid) and therefore diet is important for supplying this antioxidant vitamin. Two well

known consequences of vitamin C deficiency are the diseases scurvy and anaemia (FAO/WHO, 2004).

The antioxidant vitamin E, also previously reported in chestnut fruits, is the major lipid-soluble antioxidant in the cell and it is exclusively obtained from the diet. This vitamin acts protecting polyunsaturated fatty acids, low density lipoprotein and other cell membrane components from oxidation by free radicals and therefore reducing the risk of cancer and cardiovascular diseases. The compositional analysis of vitamins C and E in raw chestnut fruits has been reported by Peña-Mendez *et al*. (2008) and Barreira *et al*. (2009), and high levels of these vitamins are also reported in other nuts (Alasalvar *et al*., 2003).

The purpose of this study was to evaluate in detail, for the first time, the changes in composition of minerals, free sugars, antioxidant vitamins and pigments in the fruits of four different Portuguese and two other selected European cultivars of *Castanea sativa* Mill. from two harvesting seasons, during the four-stage industrial processing (fresh, stored, peeled and frozen chestnuts).

5.2 Materials and Methods

5.2.1 Plant Material and Processing

The Portuguese cultivars analyzed in the first year (2006) were Longal, Judia and Martaínha, and the two other cultivars were Spanish ("Puga do Bolo" from Galiza region) and Italian ("Viterbes" a.k.a. "Marron Vero" from Viterbo region). Only Portuguese cultivars were analyzed in the second year (2007): Longal, Judia, Martaínha and Lada. In both years the cultivars were harvested in October/November from orchards where they are mainly implanted. The samples were collected at Sortegel-Produtos Congelados S.A. (Bragança - Portugal), an enterprise involved in processing chestnut and commercialization of this fruit. The samples were collected at each one of the selected processing steps: (A) fresh at reception; (B) after storage during 3 months at ± 0 °C and RH (relative humidity) = 90%; (C) after industrial peeling by flame or fire ("brûlage") at high temperatures (800 to 1000 ºC) during 1 to 2 seconds in a rotary cylindrical oven; and (D) after freezing in a tunnel with a CO_2 flow at -65 °C during 15 to 20 minutes (for details see Supporting Information). The samples were divided into two portions. One portion (for analyses of minerals, free sugars, vitamin E and pigments) was frozen with liquid nitrogen, powdered, and freeze-dried in a Dura Dry μP from F.T.S. Systems (Stone Ridge, New York) for at least 48 h. These freeze-dried samples were ground to homogeneous fine

powders in a model 843 food processor (Moulinex, Italy).The other portion was frozen fresh and kept at -80^{o}C in a ultra-freezer until vitamin C measurements were performed.

5.2.2 Chemicals

Chemicals and reagents were of analytical grade and were obtained from a commercial source (Reagente 5, Porto-Portugal). Solvents were of high-performance liquid chromatography (HPLC) grade, and all water was ultrapure. All tocopherols (α, γ and δ), vitamin C (isoascorbic, ascorbic and dehydroascorbic acids), and pigment internal standard (trans-β-apo-8′-carotenal - APO) were obtained from a commercial source (Reagente 5). Carotenoid and chlorophyll standards were obtained from Reagente 5 and Extrasynthese (Genay, France).

5.2.3 Macro- and Micro-Minerals Composition

The mineral analyses were performed, using the freeze-dried samples, by the Department of Food Science and Technology at CEBAS-CSIC (Múrcia - Spain). Cations were extracted from 0.05g of ground dry material with 10 ml of deionised water. This extract was diluted and for analysis an ICP plasma analyser (IRIS Intrepid II XDL, Thermo Electron Corporation) was used (López-Berenguer *et al*., 2007).

5.2.4 Extraction and Ion-Exchange Chromatography Identification and Quantification of Free Sugars

Samples were analyzed using a method based on that of Bernárdez *et al*. (2004). Freeze-dried samples (100 mg), in duplicate, for each sample, were extracted with 5mL 60% ethanol and 1mL internal standard (Rhamnose, 25mg/mL) for 1 hour using a horizontal agitator followed by centrifugation (3000 rpm, 5 min, 20 ^{o}C). The supernatants were analyzed by ion-chromatography (Dionex ICS 5000) using a CarboPac P20 column with a CarboPac pre-column and detection of the sugars was done using pulse amperometry. The quantification was done by relating the recovery of the internal standard to a 100% control, recovery of added sugars in spiked samples, and calibration curves for each of the studied sugars (Fructose, Glucose, Sucrose and Maltose).

5.2.5 Carotenoids Extraction and Analysis

This method is based on one previously described (Mendes-Pinto *et al*., 2005) with minor modifications. Triplicate samples (3 x 1000mg) of the freeze-dried chestnuts were extracted and

then analyzed using a Thermo-Finnigan "Surveyor" HPLC system with the thermostatically-controlled auto-sampler set at 10 ^{o}C, and the thermostatically-controlled column oven set at 25 ^{o}C and a photo-diode array detector in combination with a Phenomenex Luna C_{18} (2) (250 x 4.6mm, 5µm) with a "Securityguard" pre-column containing a C_{18} cartridge. Different concentrations of lutein were injected to produce a calibration curve.

5.2.6 Vitamin E Extraction and Analysis

Triplicate samples (3 x 600mg; chestnuts freeze-dried and then powdered) were weighed into 10mL glass tubes and extracted with 10mL 90% v/v HPLC grade acetonitrile/10% ultra-pure water containing 1mg/mL of BHT (butylated hydroxyltoluene) as an antioxidant. Samples were vortex mixed (10 seconds) and left for 20 min at 20 ^{o}C, with vortex mixing (10 seconds) every 5 min to optimize extraction. Samples were centrifuged (4000 rpm, 20 ^{o}C, 10 min) and the supernatants transferred to 50mL round-bottom flasks. Samples were re-extracted twice more with 10mL 90% v/v HPLC grade acetonitrile/10% ultra-pure water containing 1mg/mL of BHT. The pooled extracts were rotary evaporated (water bath set at 40 ^{o}C) to a minimum volume (approximately 500µL) and the volume accurately adjusted to 1.5mL, in 2mL screw-cap micro-tubes, with 90% v/v HPLC grade acetonitrile/10% ultra-pure water containing 1mg/mL of BHT. Samples were centrifuged (13000 rpm, 4 ^{o}C, 20 min) and the upper yellow (oil) phase was carefully removed and the final volume adjusted to 500µL with 90% v/v HPLC grade acetonitrile/10% ultra-pure water containing 1mg/mL of BHT. Samples were separated on a Thermo-Finnigan Surveyor HPLC system, using a Thermo BetaBasic-8 column (100 x 4.6mm, 5µm) with a Securityguard pre-column containing a C_{18} cartridge; auto-sampler set at 10^{o}C and column oven set at 25 ^{o}C. Flow rate was 1mL/min. Solvent A was ultra-pure water and solvent B was HPLC-grade acetonitrile. A linear gradient was used: 0 min (20% A, 80% B), 30 min (20% A, 80% B), 35 min (100% B), 40 min (100% B), 45 min (20% A, 80% B). Peaks were detected by fluorescence (excitation 294nm, emission 326nm). Calibration curves were produced with various tocopherols. Inter-day variation of external standards was less than 1%.

5.2.7 Vitamin C Extraction and Analysis

This method, for the simultaneous measurement of ascorbic acid and dehydro-ascorbic acid, is based on one previously described (Martínez-Sanchez *et al*., 2006) with minor modifications. Peeled chestnut samples, in triplicate (accurately weighed, 10g) were frozen and kept at -80 ^{o}C until extraction. Frozen samples were crushed, while still frozen, and

homogenised, processed and derivatized as described by Martínez-Sanchez *et al*. (2006). Isoascorbic acid was used as the internal standard. The samples were analyzed using a Thermo-Finnigan Surveyor HPLC system, with a Thermo BetaBasic-8 column (100 x 4.6mm, 5µm) with a "Securityguard" pre-column containing a C_{18} cartridge; auto-sampler set at 5^{o}C and the column oven set at 25 ^{o}C. The flow rate of the elution solvent (methanol:water 5:95 v/v with 5mM CTAB and 5mM potassium dihydrogen phosphate pH 4.59) was 1mL/min; isocratic for 20 min. Calibration curves were generated with different concentrations of L-ascorbate and dehydroascorbate.

5.2.8 Statistical Analysis

Data were analyzed by two-way analysis of variance (ANOVA) followed by Duncan's new multiple range test with a 0.05 significance level, using the *SuperANOVA* (1.1 version, Abacus Concepts Inc., 1989) statistical package. Cultivars and processing steps were the sources of variation considered, having as error term the fixed effect of these factors. The percent contribution (Var. %) of each one of the sources (cultivar, processing stage and their interaction cultivar x processing stage) for the total variation observed in each one of the analyzed parameters was calculated as previously described by Snedecor and Cochran (1980) and Sokal and Rohlf (1981).

5.3 Results and Discussion

5.3.1 Mineral Contents

In 2006 six macro-minerals (Na, P, K, Ca, Mg and S) and six micro-minerals (Cu, Fe, Mn, Zn, B and Se) were found (Table 5.1). The same macro- and micro-minerals were found in 2007 with the exception of boron and selenium that were not determined (Table 5.2). Potassium and phosphorous were predominant and therefore these elements revealed the highest variability in absolute values, but in relative values sulphur in 2007 (CV=29.5%) and calcium either in 2006 (CV=15.3%) or 2007 (CV=17.7%) were the elements that revealed a higher variability. In 2006 the interaction cultivar x processing stage factor had more of an effect in the content variation of the majority of the minerals (Ca, K, Mg, S, Fe, Mn and B), while in 2007 this contribution was divided between the factors cultivar (Ca, K, Fe, Cu, and Mn) and processing stage (P, Mg, S, Na and Zn). The 2006 samples reveal that the majority of the contents of the minerals did not present a significant variation from stage A to B (not affected by the storage period), but there

was a significant decrease from stage B to C (Table 5.1). In 2007, the influence of the processing stages was different revealing a significant increase in the majority of the minerals from stage A to B (Table 5.2). The 2006 harvest data reveals that the highest contents of potassium were found in the "Italian" cultivar (811mg $100g^{-1}$ DM) and the highest contents of calcium and phosphorous in the "Spanish" cultivar (46 and 128mg $100g^{-1}$ DM, respectively), while there were no significant differences between the Portuguese cultivars. In 2007 the highest contents of Ca, P, K, S and Fe were found in Lada (40, 142, 805, 46 and 3,5mg $100g^{-1}$ DM, respectively), and the highest contents of Mg and Mn in Judia (58 and 5 mg $100g^{-1}$ DM, respectively) (Tables 5.1 and 5.2). Comparing 2006 and 2007 harvests, the contents of the minerals were relatively consistent with the exception of the micro-mineral Fe with a significant decrease of approximately 50%. The mineral contents found in the present study are similar to those previously obtained (Pereira-Lorenzo *et al*., 2006; Borges *et al*., 2008).

5.3.2 Free Sugars Contents

In 2006 sucrose showed a statistically significant increase from stage A to stage C. In the same year the glucose and fructose contents showed highest levels in the processing stage C, while the maltose contents were below the detection limit (Table 5.3). The increase of sucrose detected may be related to the catabolism of starch, because besides the sucrose already existing in the fruit, after the storage period starch can be enzymatically catabolized (the mechanism that precedes germination) forming maltose and glucose. In 2007 the sucrose contents increased from stage A to B, followed by a decrease from B to C. Stage B showed the highest contents of sucrose, while stage C showed the highest contents of the remaining free sugars (Table 5.3). The factors that had a bigger effect on the variation in free sugars, for both harvest years, were the interaction cultivar x processing stage and with greater influence the processing stage, especially for sucrose and maltose in 2007. Previous analyses of free sugars in raw Spanish chestnut kernels found 6.6 to 19.5g $100g^{-1}$ DM for sucrose, 0.0 to 0.3g $100g^{-1}$ DM for glucose, and 0.04 to 0.3g $100g^{-1}$ DM for fructose (Bernárdez *et al*., 2004). Free sugars in Italian chestnut kernels showed previously values in fresh material of 29.7, 1.4, 1.9 and 1.5g $100g^{-1}$ DM for sucrose, glucose, fructose and maltose, respectively (Attanasio *et al*., 2004). The presence of these sugars together with the high percentage of starch makes the sensory characteristics of chestnut kernels more pleasant to the taste (sweeter) and therefore commercially more important. The increase in free sugars from stage A to B may be related to the catabolism of starch which has previously been shown to significantly decrease from stage A to B (De Vasconcelos *et al.*, 2009a).

5.3.3 Carotenoids Contents

Example chromatograms of standards and a chestnut sample are shown in Figure 5.2 (A and B, respectively). In 2006 the highest lutein contents were found in the cultivar Judia (0.61µg g^{-1} FW) and the highest level of "total minor carotenoids" in Judia and "Spanish" cultivars (0.23µg g^{-1} FW). The highest β-carotene content was found in Martaínha (0.12µg g^{-1} FW) (Table 5.3). For all the pigments found, there was a decrease from processing stage B to C, which may be explained by the decomposition of these compounds during the peeling process. The cultivar factor and the interaction between the two factors had more of an effect on the variation in the contents of lutein and β-carotene, while for the remaining carotenoids the processing stage and the interaction had more of an effect (33.0 and 33.6%, respectively) (Table 5.3). The reduced quantity of β-carotene, when compared with the content of the "total minor carotenoids", may be related to its role in the biosynthesis of these minor carotenoids (zeaxanthin, violaxanthin and neoxanthin). In 2007 other photosynthetic pigments were detected - lutein esters. Considering all the cultivars, the lutein and the total minor carotenoids contents significantly decreased from processing stage A to C, while the lutein esters significantly decreased from A to B and from C to D. The cultivar with the highest values of lutein, β-carotene and lutein esters was Martaínha (0.25, 0.10 and 0.41µg g^{-1} FW, respectively) (Table 5.3). The factors that had more influence on the total variation were the processing stage and the interaction between the two factors. The carotenoids (lutein, β-carotene and total minor carotenoids) contents were higher in the 2006 harvest, but the lutein esters were only detectable in samples from 2007 (Table 5.3). The photosynthetic pigment lutein commonly occurs in the acylated form as lutein esters. These compounds are more stable against high temperatures and UV light than lutein (Subagio and Naofumi, 2003). Previously carotenoids have only been reported for roasted European chestnuts and only β-carotene (0.14µg g^{-1} FW) and lutein/zeaxanthin (0.13µg g^{-1} FW) were detected (USDA, 2008). Carotenoids are sensitive to light and heat and therefore the observed changes may be related to turnover during storage (stage A to stage B) and thermal instability during flame peeling and sterilization (stage C).

5.3.4 Vitamin E Contents

Example chromatograms of standards and a chestnut sample are shown in Figure 5.2 (C and D, respectively). In 2006 δ-tocopherol and γ-tocopherol significantly increased from processing stage A to B, followed by a decrease to stage C (Table 5.4). The highest values of δ-tocopherol and γ-tocopherol were found in the cultivar Longal (0.65 and 14.74µg g^{-1} FW,

respectively). The factors that had more influence on the total variation for the δ-tocopherols were the cultivar (18.5%) and the interaction (21.8%), while the γ-tocopherol content was more significantly affected by the interaction (35.9%) (Table 5.4). In 2007 the levels of γ-tocopherols were significantly higher than the δ-tocopherols, while α-tocopherol levels were below the detection limit. The highest content of δ-tocopherol was found in the cultivar Judia (1.0µg g^{-1} FW), while the values found for γ-tocopherols were not significantly different between cultivars (Table 5.4). Considering all the cultivars, there was a decrease in tocopherols from stage A to C, followed by an increase in stage D. The cultivar factor had more influence on the content of the δ-tocopherol (19.6%), while the γ-tocopherols were more affected by the processing stage factor (12.9%) (Table 5.4). A recent study analyzed the tocopherol contents in Portuguese chestnut cultivars revealing low values of α-tocopherols (between 0.02 and 0.1µg g^{-1} FW), γ-tocopherols (between 3.8 and 4.8µg g^{-1} FW) and δ-tocopherols (between 0.2 and 0.3µg g^{-1} FW) (Barreira *et al.*, 2009). The present study reveals higher values of γ-tocopherols and δ-tocopherols but neither α-tocopherol nor tocotrienols were detected.

5.3.5 Vitamin C (Ascorbic Acid and Dehydroascorbic Acid) Contents

The highest ascorbic and dehydroascorbic acid contents in 2006 were found in the cultivar Judia (6.7 and 6.1mg $100g^{-1}$ FW, respectively) (Table 5.4). Considering all the cultivars in this harvest year the highest values of ascorbic and dehydroascorbic acid were found in processing stage A (6.4 and 6.8mg $100g^{-1}$ FW, respectively), followed by a decrease to stage B. The factors studied that significantly affected the ascorbic acid contents were the cultivar (6.3%) and the processing stage (8.4%). The content of dehydroascorbic acid was more affected by processing stage factor (16.8%) (Table 5.4). In 2007 ascorbic and dehydroascorbic acid values were not significantly affected by the cultivar, while the processing stage was the measured factor that more significantly affected (14.9%) the ascorbic acid variation. The interaction between the two factors had more influence on the dehydroascorbic acid contents (12.9%) (Table 5.4). Ascorbic acid significantly decreased from stage A to B, while the dehydroascorbic acid values were not affected by the processing stage factor between these two stages. The dehydroascorbic acid content significantly decreased after the peeling process (stage C). Previously only total vitamin C (ascorbic plus dehydroascorbic acid) contents were analyzed, revealing a significant content of vitamin C (30.8 to 36.3mg $100g^{-1}$ FW) (Peña-Mendez *et al.*, 2008). The changes observed in vitamin C contents, from stages A to D, may be related to natural turnover occurring during post-harvest (pre-germination related changes) from stages A

to B and thermal effects occurring from stages B to C leading to decomposition (losses) of both ascorbic acid and dehydroascorbic acid; dehydroascorbic acid can further irreversibly degrade into 2,3-diketoglulonic acid (Chen *et al.*, 2003). At least four different biosynthetic routes for ascorbic acid have been identified in plants (Valpuesta and Botella, 2004; Hancock and Viola, 2005; Wolucka and Van Montagu, 2007). The possible precursors of ascorbic acid are sugars derived from photosynthesis or related pathways. It is possible that the free sugars in chestnut fruits could feed into ascorbic acid biosynthesis e.g. cleavage of maltose yielding two glucose molecules, cleavage of sucrose yielding fructose and glucose followed by glucose and fructose being used for ascorbic acid biosynthesis. However these sugars increase at stages were the ascorbic acid levels are decreasing and this suggests that either conversion of these sugars into ascorbic acid in the fruits is poor or that the rate of ascorbic acid decomposition (to dehydroascorbic acid and then irreversibly into 2,3-diketoglulonic acid) is faster than the rate of biosynthesis.

5.4 Conclusions

In the chestnut fruit samples from all cultivars and processing stages analysed the minerals K and P were predominant, while the minerals Cu and Zn revealed the lowest values in both harvest years. For the free sugars in both harvest years, and in all four stages, sucrose predominated with much lower levels of glucose, fructose and maltose. The factor that had more of an effect on the free sugars contents was the processing stage. In 2006 the predominant carotenoid was lutein, although other minor carotenoids were also present, while in 2007 lutein esters were predominant in all the cultivars and stages. The amount of δ and γ-tocopherols found in 2006 samples revealed a significant increase from stage A to B. The γ-tocopherols were predominant in all of the samples and the contents of δ and γ-tocopherols were higher in 2007. Overall, industrial processing had a positive effect (increase of values from stage A to D) on the tocopherols contents of both harvest years, as well on the carotenoids content of 2006. On the other hand the industrial processing had a negative effect on the vitamin C contents of both harvest years and on the carotenoids content of 2007.

Based on the results of our earlier studies (De Vasconcelos *et al*., 2009a and 2009b) and of this current paper, it is concluded that the industrial processing of chestnut fruits has both positive and negative effects in terms of composition. Some variations due to the harvest year were also observed. The positive effects include an extension of the shelf life of the fruits and increases in crude energy, fibre, phenolics, amino acids and tocopherols. The negative effects are

reductions in the levels of total starch, fat and vitamin C. It is necessary to perform further studies to evaluate possible changes in the industrial processes that will minimize the negative effects. Further studies extended to different chestnut cultivars will also be expected.

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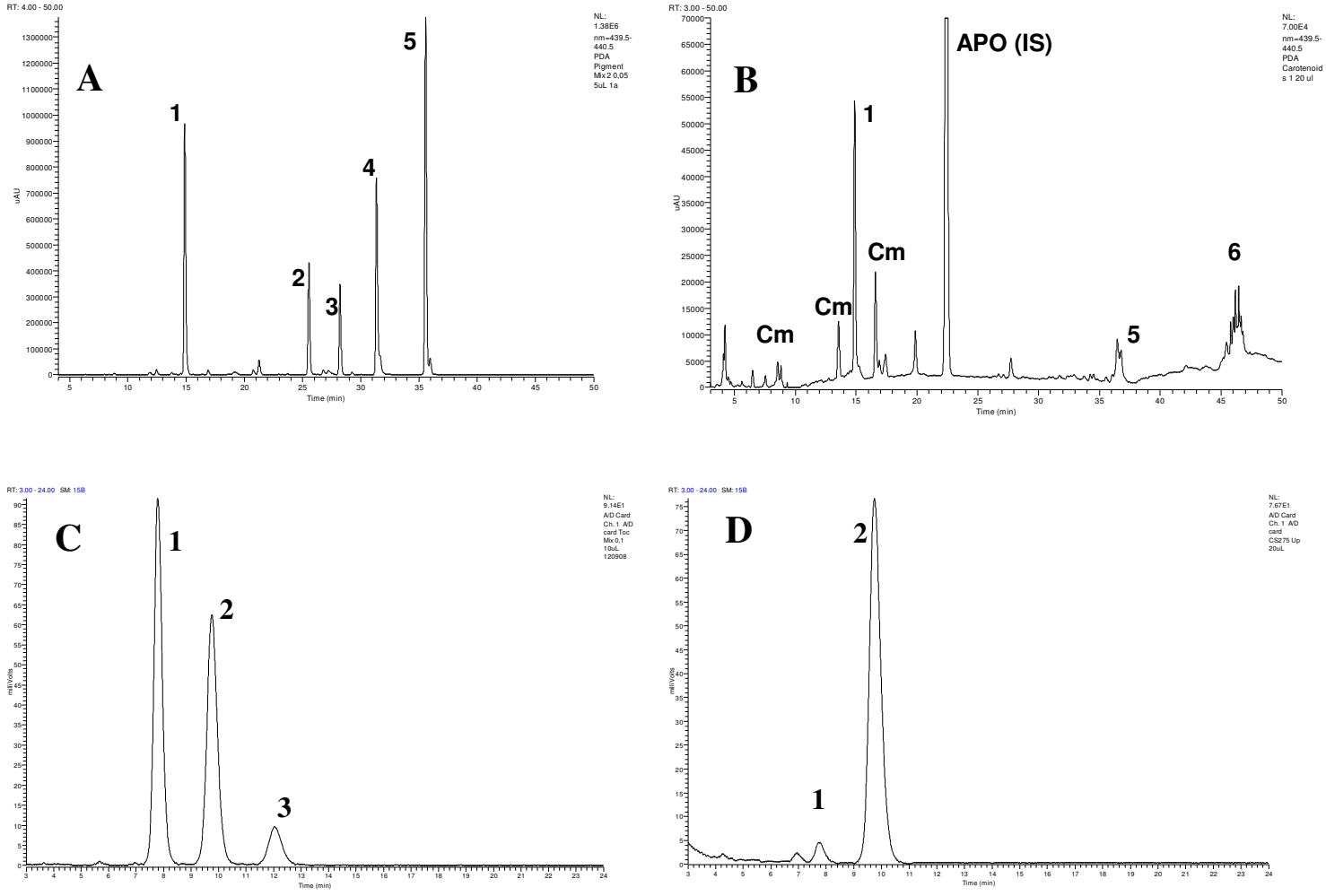

Figure 5.1 - Example chromatograms of pigments (A and B) and tocopherols (C and D).

Pigment standards (A) and a chestnut fruit extract (B): Peak ID: 1 = Lutein; 2 = Chlorophyll b; 3 = Chlorophyll a; 4 = Lycopene; 5 = Beta-caratone isomers; 6 = Lutein/Other Chlorophyll Fatty Acid Esters; Cm = Minor Carotenoids. A 10µL injection of combined tocopherol standards (0.1mg/mL each tocopherol) (C) and 20µL injection of a chestnut fruit extract (D). Data shown are from the fluorescence signals, and each chromatogram is in full scale. Peak ID: 1 = γ-tocopherol, 2 = δ-tocopherol, 3 = α-tocopherol.

Table 5.1 - Effect of cultivar, processing step and respective interaction on mineral composition (mg $100g^{-1}$ DM) of raw shelled chestnut kernel samples (edible portion) of 2006 harvest [(1)].

	CULTIVAR							PROCESSING STEP						INTERACTION	
	Judia	**Longal**	**Martaínha**	**"Italian"**	**"Spanish"**	P	Var.	**A**	**B**	**C**	**D**	P	Var.	**C*PS**	
Minerals							(%)						(%)	P	Var. (%)
Ca	35.7±8.0 [a]	38.6±5.1 [b]	37.2±2.3 [ab]	39.2±3.1 [b]	45.8±4.8 [c]	0.000	13.5	41.4±7.5 [c]	37.6±5.9 [a]	39.9±5.9 [bc]	38.3±4.0 [ab]	0.001	0.0	0.000	41.9
P	109.8±3.5 [a]	121.2±4.4 [b]	120.8±4.9 [b]	109.8±1.4 [a]	123.7±10.5 [c]	0.000	38.6	121.1±8.7 [b]	119.6±10.5 [b]	114.5±4.9 [a]	112.9±5.0 [a]	0.000	10.9	0.000	19.8
K	640.4±23.3 [a]	633.4±36.5 [a]	633.8±49.5 [a]	811.1±86.2 [c]	694.2±151.2 [b]	0.000	30.6	749.5±91.9 [c]	722.3±109.2 [c]	629.2±50.4 [a]	629.3±107.9 [a]	0.000	19.6	0.000	31.5
Mg	49.4±2.1 [a]	53.1±3.2 [b]	57.0±4.3 [c]	57.4±3.7 [c]	57.5±5.2 [c]	0.000	24.8	56.8±6.2 [b]	55.7±5.6 [b]	53.9±3.0 [a]	53.1±3.8 [a]	0.000	0.0	0.000	38.4
S	36.8±1.7 [b]	36.8±1.7 [b]	36.4±1.5 [ab]	35.5±0.9 [a]	35.5±1.5 [a]	0.013	0.0	36.2±1.8 [a]	36.1±1.3 [a]	36.3±1.8 [a]	36.2±1.3 [a]	0.969	0.0	0.001	18.2
Na	27.0±1.5 [a]	26.9±1.2 [a]	26.7±0.6 [a]	28.3±1.0 [b]	28.0±1.8 [b]	0.000	8.7	26.7±1.0 [a]	26.4±0.9 [a]	28.4±1.4 [b]	28.1±1.2 [b]	0.000	21.1	0.010	8.6
Fe	7.1±0.3 [a]	6.9±0.2 [a]	7.0±0.3 [a]	7.0±0.2 [a]	6.9±0.3 [a]	0.312	0.0	7.1±0.3 [b]	6.9±0.2 [b]	6.8±0.2 [a]	7.1±0.2 [b]	0.001	5.6	0.003	14.3
Cu	0.9±0.2 [c]	0.9±0.1 [c]	0.7±0.2 [ab]	0.8±0.1 [bc]	0.7±0.1 [a]	0.005	5.8	0.8±0.2 [a]	0.8±0.1 [a]	0.8±0.2 [a]	0.8±0.2 [a]	0.782	0.0	0.205	3.4
Zn	0.8±0.1 [a]	0.9±0.1 [a]	0.8±0.1 [a]	0.9±0.2 [a]	0.8±0.2 [a]	0.181	1.4	0.9±0.2 [a]	0.8±0.1 [a]	0.9±0.2 [a]	0.8±0.1 [a]	0.692	0.0	0.475	0.0
Mn	3.2±0.3 [c]	2.0±1.1 [a]	3.2±0.5 [c]	2.3±0.5 [b]	3.3±0.2 [c]	0.000	34.8	3.3±0.7 [c]	2.5±1.0 [a]	2.8±0.7 [b]	2.6±0.7 [a]	0.000	4.0	0.000	41.4
B	3.1±0.1 [b]	3.1±0.1 [b]	3.1±0.1 [b]	3.0±0.1 [a]	3.0±0.1 [a]	0.013	0.0	3.1±0.1 [a]	3.0±0.1 [a]	3.1±0.1 [a]	3.0±0.1 [a]	0.790	0.0	0.001	17.6
Se	0.5±0.2 [a]	0.7±0.3 [a]	0.7±0.3 [a]	0.7±0.3 [a]	0.6±0.4 [a]	0.177	1.5	0.6±0.2 [ab]	0.8±0.3 [b]	0.4±0.3 [a]	0.5±0.3 [a]	0.004	7.7	0.507	0.0

[(1)] Tabulated values are sample means ± standard deviation (SD) of mean.

C - Cultivar; PS - Processing stage; Var. % - percent contribution for the total variation observed.

Within each row and for each one of the factors considered, means with a different letter are significantly different (P<0.05), according to Duncan New Multiple Range Test.

Table 5.2 - Effect of cultivar, processing step and respective interaction on mineral composition (mg $100g^{-1}$ DM) of raw shelled chestnut kernel samples (edible portion) of 2007 harvest [(1)].

	CULTIVAR						PROCESSING STEP						INTERACTION	
	Judia	**Longal**	**Martaínha**	**Lada**	P	Var.	**A**	**B**	**C**	**D**	P	Var.	**C*PS**	
Minerals						(%)						(%)	P	Var. (%)
Ca	40.2±3.5 ^{c}	30.8±3.8 ^{a}	34.9±7.5 ^{b}	40.3±5.2 ^{c}	0.000	31.3	31.4±5.2 ^{a}	33.9±4.7 ^{b}	40.2±6.5 ^{c}	40.8±4.0 ^{c}	0.000	32.6	0.001	0.0
P	132.5±26.9 ^{b}	133.1±23.5 ^{b}	124.6±21.3 ^{a}	142.3±19.4 ^{c}	0.000	6.4	99.8±10.1 ^{a}	130.2±6.0 ^{b}	146.2±7.4 ^{c}	156.4±9.3 ^{d}	0.000	81.1	0.014	1.7
K	796.0±38.2 ^{c}	745.5±16.1 ^{b}	703.5±33.9 ^{a}	804.9±38.5 ^{c}	0.000	40.4	730.6±37.9 ^{a}	778.5±62.6 ^{b}	764.3±42.7 ^{b}	776.6±53.6 ^{b}	0.000	7.7	0.191	2.1
Mg	57.5±3.1 ^{b}	57.4±3.3 ^{b}	57.6±2.4 ^{b}	52.8±1.7 ^{a}	0.000	23.1	53.1±2.5 ^{a}	56.4±3.2 ^{b}	57.0±2.5 ^{b}	58.7±2.6 ^{c}	0.000	23.7	0.240	1.6
S	40.4±15.3 ^{b}	40.6±9.9 ^{b}	35.1±10.0 ^{a}	45.9±10.7 ^{c}	0.002	5.1	26.4±5.9 ^{a}	37.4±8.2 ^{b}	47.8±6.4 ^{c}	50.3±8.7 ^{c}	0.000	40.3	0.173	2.4
Na	21.4±2.6 ^{a}	21.4±2.8 ^{a}	22.2±2.2 ^{a}	21.8±2.4 ^{a}	0.627	0.3	19.9±1.5 ^{a}	19.9±1.5 ^{a}	22.6±1.4 ^{b}	24.5±1.5 ^{c}	0.000	32.0	0.924	0.0
Fe	3.1±0.2 ^{a}	3.0±0.2 ^{a}	3.0±0.2 ^{a}	3.5±0.2 ^{b}	0.000	25.1	3.1±0.3 ^{a}	3.2±0.3 ^{a}	3.2±0.3 ^{a}	3.2±0.3 ^{a}	0.326	1.3	0.943	0.0
Cu	0.8±0.1 ^{b}	0.8±0.1 ^{b}	0.6±0.1 ^{a}	0.8±0.0 ^{b}	0.000	45.3	0.7±0.1 ^{a}	0.8±0.1 ^{b}	0.7±0.1 ^{b}	0.8±0.1 ^{b}	0.001	5.7	0.151	3.8
Zn	0.9±0.3 ^{a}	1.0±0.3 ^{a}	1.0±0.4 ^{a}	1.1±0.3 ^{a}	0.159	1.7	0.6±0.1 ^{a}	1.0±0.2 ^{b}	1.0±0.3 ^{b}	1.3±0.2 ^{c}	0.000	29.6	0.744	0.0
Mn	5.1±0.4 ^{c}	1.5±0.6 ^{a}	2.7±0.4 ^{b}	2.6±0.2 ^{b}	0.000	83.7	3.0±1.1 ^{a}	2.9±1.7 ^{a}	3.0±1.6 ^{a}	3.0±1.3 ^{a}	0.256	0.0	0.000	5.6
B	ND	ND	ND	ND			ND	ND	ND	ND				
Se	ND	ND	ND	ND			ND	ND	ND	ND				

[(1)] Tabulated values are sample means ± standard deviation (SD) of mean.

ND - not determined; C - Cultivar; PS - Processing stage; Var. % - percent contribution for the total variation observed.

Within each row and for each one of the factors considered, means with a different letter are significantly different (P<0.05), according to Duncan New Multiple Range Test.

Table 5.3 - Effect of cultivar, processing step and respective interaction on free sugars (g $100g^{-1}$ DM) and carotenoids content (µg g^{-1} FW) of raw shelled chestnut kernel samples (edible portion) of 2006 and 2007 harvests [(1)].

	CULTIVAR							PROCESSING STAGE						INTERACTION	
2006	**Judia**	**Longal**	**Martaínha**	**"Italian"**	**"Spanish"**	P	Var. (%)	**A**	**B**	**C**	**D**	P	Var. (%)	**C*PS** P	**C*PS** Var. (%)
Free sugars															
Sucrose	11.32±1.50 ^{b}	10.62±1.94 ^{a}	11.35±1.27 ^{b}	10.35±3.32 ^{a}	10.27±2.00 ^{a}	0.000	0.0	8.82±1.74 ^{a}	9.26±1.47 ^{b}	12.39±0.46 ^{c}	12.70±0.79 ^{c}	0.000	59.5	0.000	27.8
Glucose	0.17±0.16 ^{c}	0.16±0.17 ^{c}	0.12±0.10 ^{b}	0.07±0.05 ^{a}	0.22±0.13 ^{d}	0.000	7.4	0.06±0.05 ^{a}	0.08±0.09 ^{a}	0.28±0.14 ^{c}	0.15±0.10 ^{b}	0.000	40.0	0.000	35.6
Fructose	0.21±0.25 ^{ab}	0.25±0.35 ^{b}	0.15±0.15 ^{ab}	0.11±0.13 ^{a}	0.18±0.09 ^{ab}	0.165	0.0	0.07±0.04 ^{a}	0.10±0.11 ^{a}	0.41±0.27 ^{b}	0.12±0.07 ^{a}	0.000	22.6	0.023	8.4
Maltose	T	T	T	T	T			T	T	T	T				
Pigments															
Lutein	0.61±0.18 ^{c}	0.35±0.09 ^{a}	0.39±0.14 ^{a}	0.38±0.05 ^{a}	0.55±0.18 ^{b}	0.000	26.8	0.40±0.15 ^{a}	0.55±0.24 ^{c}	0.40±0.08 ^{a}	0.45±0.12 ^{b}	0.000	7.7	0.000	31.6
β-Carotene	0.06±0.04 ^{ab}	0.05±0.03 ^{a}	0.12±0.05 ^{d}	0.07±0.01 ^{b}	0.10±0.05 ^{c}	0.000	29.1	0.05±0.03 ^{a}	0.10±0.05 ^{c}	0.07±0.03 ^{b}	0.11±0.05 ^{c}	0.000	14.6	0.000	29.7
Total Minor Carotenóids	0.23±0.09 ^{b}	0.17±0.09 ^{a}	0.17±0.14 ^{a}	0.21±0.12 ^{ab}	0.23±0.16 ^{b}	0.002	3.1	0.09±0.05 ^{a}	0.27±0.15 ^{c}	0.14±0.05 ^{b}	0.30±0.08 ^{c}	0.000	33.0	0.000	33.6
2007	**Judia**	**Longal**	**Martaínha**	**Lada**		P	Var. (%)	**A**	**B**	**C**	**D**	P	Var. (%)	**C*PS** P	**C*PS** Var. (%)
Free sugars															
Sucrose	11.14±2.33 ^{b}	12.98±2.31 ^{c}	10.18±2.75 ^{a}	13.71±2.62 ^{d}		0.000	0.0	9.17±2.67 ^{a}	14.90±2.32 ^{d}	10.87±0.72 ^{b}	13.06±0.58 ^{c}	0.000	64.1	0.000	23.9
Glucose	0.22±0.29 ^{bc}	0.19±0.27 ^{b}	0.27±0.34 ^{c}	0.11±0.15 ^{a}		0.000	6.6	0.05±0.06 ^{a}	0.04±0.06 ^{a}	0.62±0.21 ^{b}	0.08±0.05 ^{a}	0.000	44.8	0.000	31.9
Fructose	0.19±0.22 ^{a}	0.24±0.35 ^{a}	0.41±0.59 ^{b}	0.24±0.31 ^{a}		0.026	0.0	0.05±0.05 ^{a}	0.08±0.07 ^{a}	0.84±0.41 ^{b}	0.12±0.02 ^{a}	0.000	25.2	0.066	7.5
Maltose	0.37±0.31 ^{a}	0.50±0.53 ^{b}	0.46±0.63 ^{b}	0.63±0.71 ^{c}		0.000	0.4	0.11±0.06 ^{a}	0.11±0.05 ^{a}	1.36±0.37 ^{c}	0.37±0.10 ^{b}	0.000	84.9	0.000	9.8
Pigments															
Lutein	0.15±0.23 ^{a}	0.23±0.14 ^{ab}	0.25±0.22 ^{b}	0.22±0.10 ^{ab}		0.155	1.2	0.37±0.18 ^{c}	0.23±0.17 ^{b}	0.14±0.09 ^{a}	0.09±0.04 ^{a}	0.000	22.3	0.000	24.7
β-Carotene	0.01±0.02 ^{a}	0.06±0.04 ^{b}	0.10±0.06 ^{c}	0.06±0.03 ^{b}		0.000	7.4	0.08±0.06 ^{c}	0.07±0.05 ^{bc}	0.05±0.03 ^{b}	0.01±0.01 ^{a}	0.000	13.0	0.004	7.4
Total Minor Carotenoids	0.03±0.06 ^{a}	0.07±0.05 ^{b}	0.10±0.10 ^{c}	0.11±0.05 ^{c}		0.000	8.6	0.15±0.07 ^{c}	0.10±0.07 ^{b}	0.03±0.03 ^{a}	0.02±0.02 ^{a}	0.000	33.4	0.000	19.1
Lutein Esters	0.12±0.17 ^{a}	0.31±0.12 ^{b}	0.41±0.22 ^{c}	0.28±0.13 ^{b}		0.000	11.1	0.43±0.17 ^{c}	0.29±0.19 ^{b}	0.27±0.11 ^{b}	0.10±0.08 ^{a}	0.000	25.2	0.001	15.4

[(1)] Tabulated values are sample means ± standard deviation of mean (SD). C - Cultivar; PS - Processing stage; Var. % - percent contribution for the total variation observed.

Within each row and for each one of the factors considered, means with a different letter are significantly different (P<0.05), according to Duncan New Multiple Range Test.

Table 5.4 - Effect of cultivar, processing step and respective interaction on vitamin E (tocopherols) and C (ascorbic and dehydroascorbic acid) content of raw shelled chestnut kernel samples (edible portion) of 2006 and 2007 harvests.

	CULTIVAR							PROCESSING STAGE						INTERACTION	
2006															
	Judia	**Longal**	**Martaínha**	**"Italian"**	**"Spanish"**	P	Var. (%)	**A**	**B**	**C**	**D**	P	Var. (%)	**C*PS** P	**C*PS** Var. (%)
δ-Tocopherol[(1)]	0.46±0.40 [b]	0.65±0.25 [c]	0.19±0.13 [a]	0.40±0.13 [b]	0.25±0.21 [a]	0.000	18.5	0.28±0.14 [a]	0.59±0.24 [c]	0.27±0.19 [a]	0.40±0.41 [b]	0.000	12.3	0.000	21.8
γ-Tocopherol[(1)]	13.91±15.59 [b]	14.74±11.18 [b]	4.14±2.43 [a]	13.82±4.31 [b]	8.17±7.84 [a]	0.000	8.6	9.39±6.94 [a]	15.90±8.10 [b]	6.20±5.37 [a]	10.73±15.90 [a]	0.000	7.2	0.000	35.9
Ascorbic Acid[(2)] (ASC)	6.74±1.98 [b]	4.71±1.13 [a]	5.39±0.77 [a]	4.72±1.63 [a]	5.24±1.89 [a]	0.004	6.3	6.44±1.44 [c]	5.38±1.98 [b]	4.20±1.14 [a]	5.42±1.34 [b]	0.001	8.4	0.475	0.0
Dehydroascorbic[(2)] Acid (DHASC)	6.13±2.72 [d]	4.66±1.82 [bc]	5.49±2.21 [cd]	4.04±1.64 [ab]	3.35±1.99 [a]	0.000	8.2	6.80±1.93 [c]	4.15±2.00 [ab]	4.73±2.08 [b]	3.24±1.49 [a]	0.000	16.8	0.046	6.0
2007															
	Judia	**Longal**	**Martaínha**	**Lada**		P	Var. (%)	**A**	**B**	**C**	**D**	P	Var. (%)	**C*PS** P	**C*PS** Var. (%)
δ-Tocopherol[(1)]	1.00±0.31 [b]	0.66±0.24 [a]	0.61±0.20 [a]	0.51±0.16 [a]		0.000	19.6	0.83±0.29 [b]	0.71±0.37 [b]	0.52±0.17 [a]	0.72±0.26 [b]	0.004	6.5	0.090	5.1
γ-Tocopherol[(1)]	22.95±7.52 [a]	21.38±5.96 [a]	22.72±9.03 [a]	22.82±7.03 [a]		0.902	0.0	25.24±5.52 [bc]	21.10±5.96 [b]	16.23±3.31 [a]	27.31±8.34 [c]	0.000	12.9	0.132	4.7
Ascorbic Acid[(2)] (ASC)	5.04±1.63 [a]	6.09±1.15 [a]	5.76±2.14 [a]	5.67±0.83 [a]		0.201	1.1	7.17±1.66 [b]	5.53±0.65 [a]	4.75±1.28 [a]	5.11±1.15 [a]	0.000	14.9	0.325	1.4
Dehydroascorbic[(2)] Acid (DHASC)	5.00±2.35 [a]	4.66±1.87 [a]	4.25±1.83 [a]	4.39±2.32 [a]		0.676	0.0	5.33±2.08 [b]	5.57±2.31 [b]	3.08±1.43 [a]	4.31±1.48 [ab]	0.002	8.4	0.011	12.9

[(1)] Data are presented as means ± standard deviation of mean (SD) μg g^{-1} fresh weight (FW). [(2)] Data are presented as means ± SD mg $100g^{-1}$ fresh weight (FW).

C - Cultivar; PS - Processing stage; Var. % - percent contribution for the total variation observed.

Within each row and for each one of the factors considered, means with a different letter are significantly different (P<0.05), according to Duncan New Multiple Range Test.

5.5 Supporting Information

The Portuguese cultivars analyzed in the first year (2006) were Longal, Judia and Martaínha, and the two foreign cultivars were Spanish ("Puga do Bolo" from Galiza region) and Italian ("Viterbes" or "Marron Vero" from Viterbo region). Only Portuguese cultivars were analyzed in the second year (2007), and these were Longal, Judia, Martaínha and Lada. In both years the cultivars were harvested in October/November from orchards where they are mainly implanted. The samples were collected at Sortegel-Produtos Congelados S.A. (Bragança - Portugal), an enterprise involved in processing chestnut and commercialization of this fruit, as both fresh (19%) and frozen (74%) kernels, and also the major company in this sector in Portugal, recently equipped with state-of-the-art machinery for the most efficient processing of chestnut fruits. The samples were collected at the end of each one of the following selected processing steps (Figure 1): (A) fresh at reception; (B) after storage during 3 months at ±0 °C and relative humidity (RH) = 90%; (C) after industrial peeling by flame or fire ("brûlage") at high temperatures (800 to 1000 ºC) during 1 to 2 seconds in a rotary cylindrical oven; and (D) after freezing in a tunnel with a CO_2 flow at -65 °C during 15 to 20 minutes. For each one of the cultivars at each of the four processing stages, three samples of 1.5kg each were collected. The samples from stages A and B were stored in a refrigerator at ±2 °C, for a maximum of 3 days until they were hand-peeled, while the samples from stage C were processed immediately, and samples from stage D were kept at -20 °C until analyzed. The samples from all the processing stages were processed either by air-drying or freeze-drying for different analysis to be performed. The raw shelled samples were divided into two portions. One portion (for analyses of minerals, free sugars, vitamin E and pigments) was frozen with liquid nitrogen, powdered, and freeze-dried in a Dura Dry μP from F.T.S. Systems (Stone Ridge, New York) for at least 48 h. These freeze-dried samples were ground to homogeneous fine powders in a model 843 food processor (Moulinex, Italy).The other portion was frozen fresh and kept at -80^{o}C in a ultra-freezer until vitamin C measurements were performed.

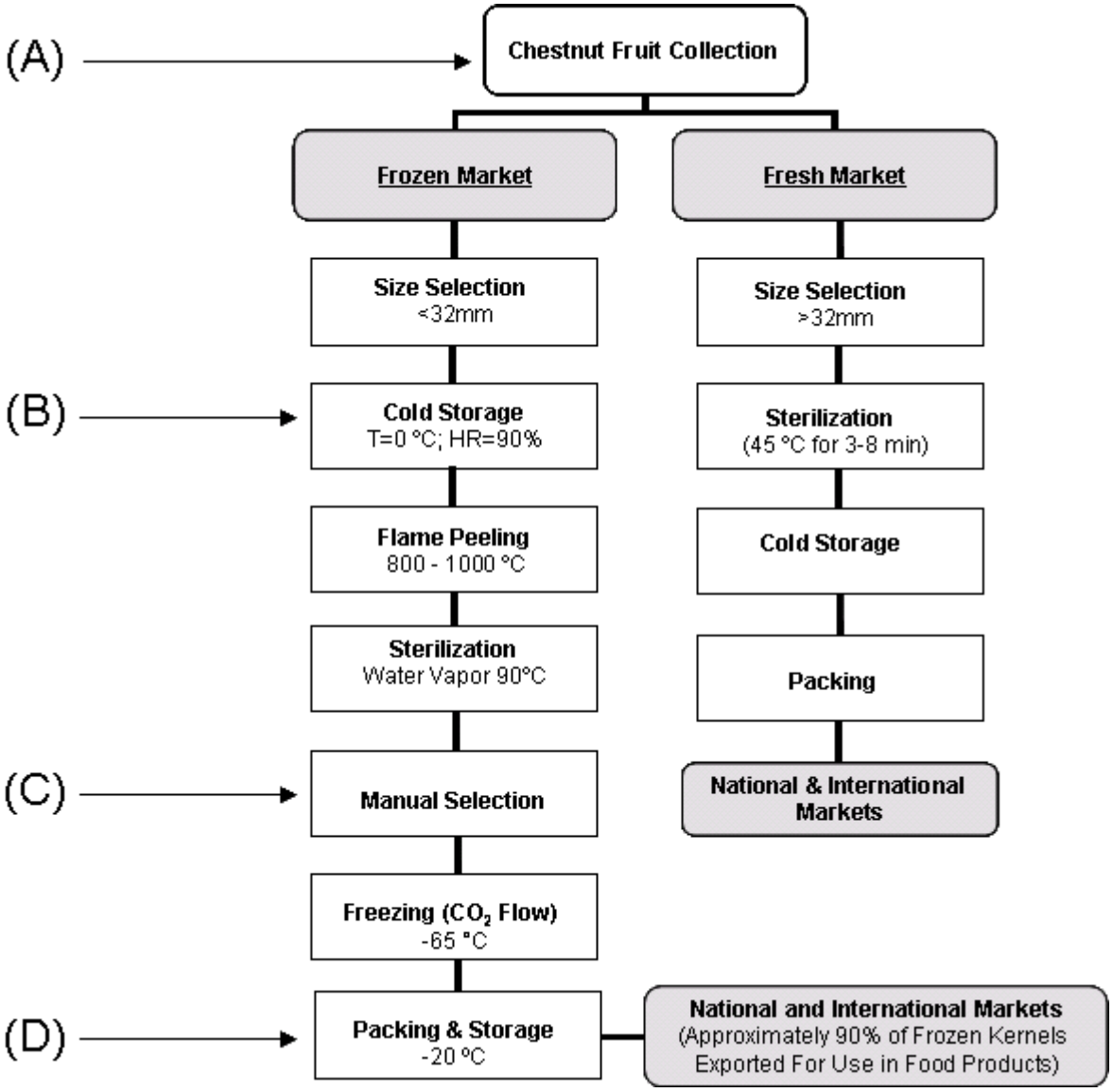

Figure 5.2 - SORTEGEL processing scheme with sampling stages (A, B, C and D) indicated.

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CHAPTER 6

The chestnut fruit processing generates large amounts of residues as pericarp (outer shell; 8.9 to 13.5%) and integument (inner shell; 6.3 to 10.1%). These materials clearly have the potential as sources of valuable co-products. The analyses of the pericarp and integument of four Portuguese chestnut cultivars (Judia, Longal, Martaínha and Lada) revealed significant contents of total phenolics, low molecular weight phenolics (gallic and ellagic acid), condensed tannins and ellagitannins including castalagin, vescalagin, acutissimin A and acutissimin B. The integument tissues had the highest levels of total phenolics and condensed tannins. The most efficient extraction solvent for the total phenolics, total condensed tannins and low molecular weight phenolics (in Longal) was 70:30 acetone:water at 20ºC. The pericarp and integument tissues of the cultivar Longal were richest in gallic acid and castalagin. It is clear that these materials could be used for the extraction of valuable phenolics.

Keywords: *Castanea sativa*; pericarp; integument; phenolics; ellagitannins; condensed tannins.

Evaluating the Potential of Chestnut (*Castanea sativa* Mill.) Fruit Pericarp and Integument as a Source of Tocopherols, Pigments and Polyphenols

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6.1 Introduction

In Portugal the cultivation of chestnut trees is mainly for nut production, either for internal consumption or export, predominantly to Spain, Italy, France, Brazil, United Kingdom, United States and Switzerland (De Vasconcelos *et al*., 2007). From the nut production a large amount of inedible waste material (Table 6.1) is generated - the pericarp (outer shell) and integument (inner shell) that surrounds the edible nuts.

Table 6.1 - Percentage of the waste material (shells) in the stages A and B of chestnut fruit industrial processing.

	Residues (% of Fresh Weight of Whole Fruit)		
Cultivars	**Pericarp**	**Integument**	**Total**
Longal A	8.96	9.82	18.78
Longal B	12.40	6.33	18.73
Judia A	10.09	10.10	20.19
Judia B	12.09	8.44	20.53
Martaínha A	10.25	8.73	18.98
Martaínha B	13.54	8.01	21.55
Lada A	12.66	8.44	21.10
Lada B	12.86	6.90	19.76

Development of low-cost efficient processing methods for these residues could lead to the generation of valuable co-products, reduce the overall fruit processing costs, and thus increase the competitiveness and economic profits for chestnut processing companies. Chestnut fruits have a long history of reported health effects related to their nutritional composition (excellent energy source due to its high starch content), specifically the absence of gluten (therefore making them ideal for preparing gluten-free foods), and the presence of unsaturated fatty acids (a good source of omega-3 fatty acids with low saturated fat contents), vitamins E and C. Not only as a good food resource, the chestnut trees can also be used as a source of timber for

construction (furniture, agronomic utensils, cooperage, and even as a source of fuel). They are also a rich source of tannins that in the past were used to produce some medicines and also play a part in the flavouring of various types of wines i.e. the tannins from the barrels are released into the wine during storage (Ferreira-Cardoso, 2002).

Industries generate waste materials and the responsibility for a sustainable agriculture implies reduction/elimination of residues that cause environmental pollution. There are great possibilities for utilizing chestnut waste materials as sources of valuable co-products and therefore decreasing the negative impacts of these wastes. This recovery is especially relevant for the food industries where the quantities of biomass resulting from the processing can be ecologically important (Laufenberg *et al*., 2003). The use of the chestnut pericarp (outer shell; husk) and integument (inner shell; pellicle) co-products could be of high economic and nutritional value because they are potentially a rich source of compounds with biological activity, e.g. antioxidant polyphenols. The intensive research about the effects of dietary polyphenols on human health in the last few years strongly supports a role for polyphenols in the prevention of degenerative diseases, especially cardiovascular diseases and cancers (Scalbert *et al*., 2005).

Previous studies on chestnut wood and on the brandies aged in chestnut wood barrels showed a high level of low molecular weight phenolics, e.g. gallic acid (Canas *et al*., 1999). Lampire *et al*. (1998) isolated eight phenolic compounds from chestnut bark: castalin, castalagin, vescalagin, kurigalin, 5-O-galloylhamamelose, (3',5'-dimethoxy-4'-hydroxyphenol)-1-O-β-D-(6-O-galloyl)glucose, chestanin, and acutissimin A (Figure 6.1). The antioxidant potential of chestnut leaves has also been evaluated showing a high level of total phenolic compounds and corresponding antioxidant effects (Calliste *et al*., 2005). Other studies have analysed the content of constitutive levels of hydrolysable tannins and proanthocyanidins in the leaves and stems of American (*Castanea dentata* Marshal) and Chinese (*Castanea molissima* Blume) chestnuts, and showed that the American chestnut contained more proanthocyanidins in leaves and stems, while the Chinese chestnut had a higher content of hydrolysable tannins in the leaves (Cooper and Rieske, 2008). Some of these tannins have also been reported in various *Quercus* species (Fridrich *et al*., 2008; Rocha-Guzmán *et al*., 2009). In terms of biological effects there have been many papers on ellagitannins, and more recently on the positive health effects of ellagitannins from pomegranates (Quideau and Feldman, 1996; Cerdá *et al*., 2004; Quideau, 2009). It is likely that some of the tannins, ellagitannins and condensed tannins, in chestnut tissues will also have beneficial health effects. There are only a few studies on the bioactives composition of the pericarp and integument of chestnuts.

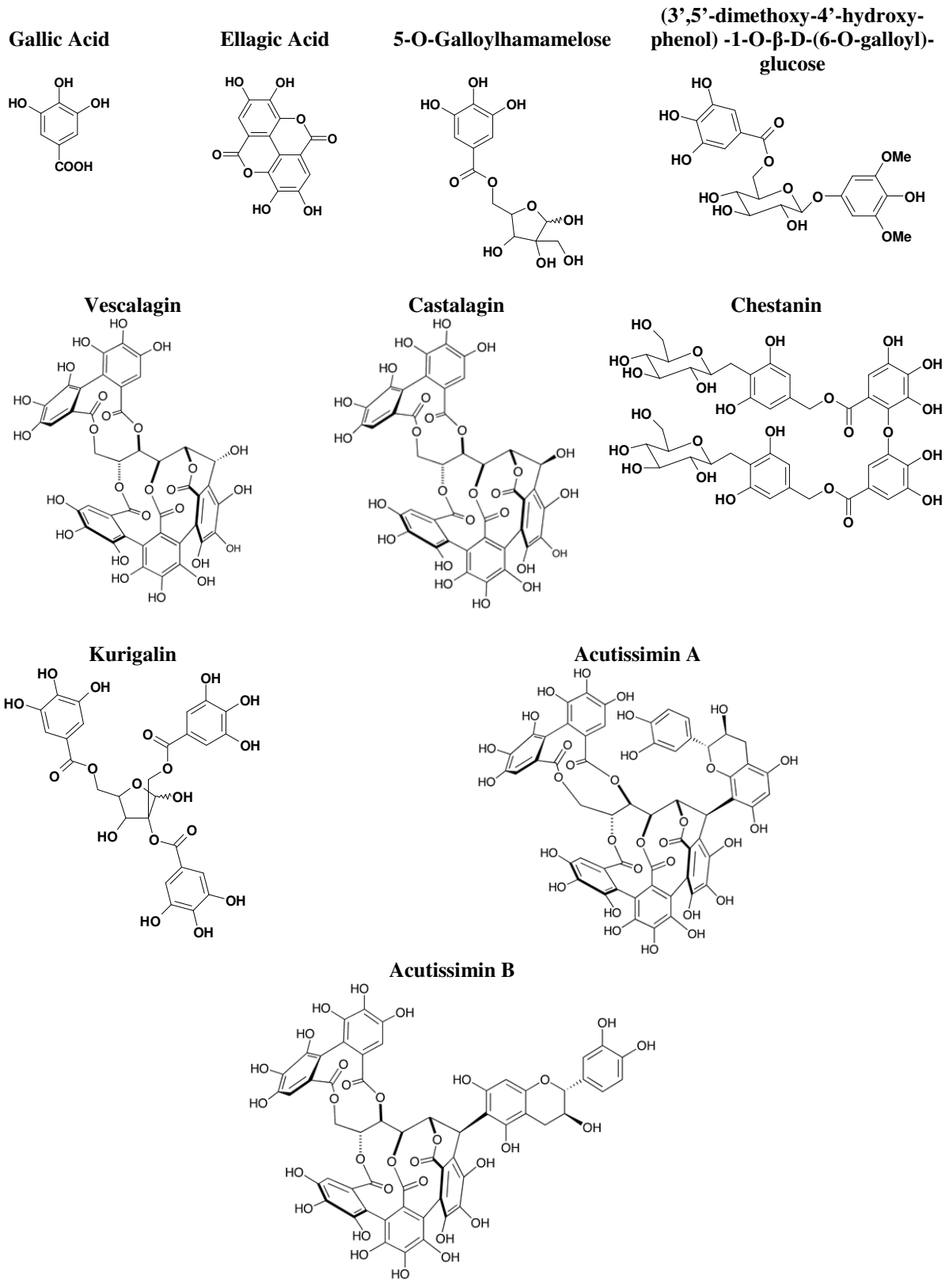

Figure 6.1 - Chemical structures of major phenolics previously identified in chestnut tissues.

A previous study on the chestnut shell (pericarp) revealed a high content of total phenolics as well as a good antioxidant activity in comparison with the results from the same analysis performed with eucalyptus bark samples (Vázquez *et al*., 2009). Barreira *et al*. (2008) analysed the phenolic contents in chestnut leaves, flowers, skins and fruits. The various chestnut tissues (leaves, wood, fruit and shell) show great potential as a source of bioactive phenolics, specifically ellagitannins and condensed tannins. Extracts from chestnut tissues could be used as phenol substitutes in the formulation of adhesives, as chrome substitutes in leather tanning and even as antioxidant supplements in food (Vázquez *et al*., 2008 and 2009). Other potential applications of the phenolics include their use as functional foods ingredients as nutraceuticals and antimicrobial agents (Chitwood, 2002; Nohynek *et al*., 2006; Thring *et al*., 2006; Espín *et al*., 2007).

This study was performed to determine the levels and profiles of tocopherols, pigments (carotenoids and chlorophylls) and specific phenolics (simple phenolics, ellagitannins and condensed tannins) in the pericarps and integuments of four Portuguese chestnut cultivars, pre- and post-storage, and thus determine the potential of these waste materials as sources of useful phytochemicals. The extraction efficiencies, for the phenolics, using different solvents was done as part of the evaluation for potential use of low cost extraction solvents for industrial processing of these wastes.

6.2 Materials and Methods

6.2.1 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 water was ultrapure. Gallic and ellagic acids and (-)-epicatechin standards were obtained from Sigma-Aldrich. All tocopherols (alpha, gamma and delta) were obtained from Sigma/Aldrich. All carotenoid and chlorophyll standards and trans-β-apo-8′-carotenal were obtained from Sigma/Aldrich. Hydrolysable tannin standards (castalagin, vescalagin, acutissimins A and B) were either extracted and purified from *Quercus* tissues or hemi-synthesized by the Quideau group (Univ. Bordeaux, France) (Quideau *et al*., 2003, 2004 and 2005). For all standards, HPLC calibration curves were constructed by injection of different stock concentrations of the standards.

6.2.2 Plant Material

The pericarp and integument tissues were obtained from the Portuguese chestnut cultivars Longal, Judia, Martaínha and Lada; these were harvested in October/November 2007 from orchards around Bragança, North East Portugal. The samples were collected at Sortegel-Produtos Congelados S.A., the major Portuguese company involved in processing chestnut fruits and its commercialization, as both fresh (19% of total fruit production) and frozen kernels (74% of total fruit production). The samples were collected at the reception/calibration stage (A - fresh) and after storage for 3 months at ±0 °C (B; average of the estimated time chestnut fruits are stored before processing, because of the large quantity of chestnuts that arrive after collection). After the collection of each sample of the four cultivars at the respective processing stage, three subsamples were taken and stored in a refrigerator at ±2 °C, for a maximum of 3 days until they were hand-peeled - separating the tissues into pericarp and integument samples, and kernel samples (data for which has been presented in a previous paper - De Vasconcelos *et al*., 2010). The pericarp and integument tissues obtained after the hand peeling were weighed (fresh weight) and freeze-dried in a Dura Dry μP from F.T.S. Systems (Stone Ridge, New York) for 48 h, weighed (dry weight), and powdered using a model 843 food processor (Moulinex, Italy).

6.2.3 Extraction and HPLC Analysis of Vitamin E (Tocopherols and Tocotrienols)

Triplicate samples (3 x 600mg; chestnuts freeze-dried and then powdered) were weighed into 10mL glass tubes. Each sample was extracted with 10mL 90% v/v HPLC grade acetonitrile / 10% ultra-pure water containing 1mg / mL of BHT (butylated hydroxytoluene) as an antioxidant. Samples were vortex mixed and left for 20 minutes at 20^{o}C, with vortex mixing every 5 min to optimize extraction. Samples were centrifuged (4,000 rpm, 20 ºC, 10 min) and the supernatants transferred to 50 mL round-bottom flasks. Samples were re-extracted twice with 10 mL 90% v/v HPLC grade acetonitrile/10% ultra-pure water containing 1 mg / mL of BHT; pooling the supernatants for each sample after each extraction. The pooled extracts were evaporated (water bath set at 40 ^{o}C) to a minimum volume (approximately 500 µL) and the volume accurately adjusted to 1.5 mL, in 2 mL screw-cap micro-tubes, with 90% v/v HPLC grade acetonitrile/10% ultra-pure water containing 1 mg / mL of BHT. Samples were centrifuged (13,000 rpm, 4 ^{o}C, 20 min) and the upper yellow (oil) phase was carefully removed and the final volume adjusted to 500 µL with 90% v/v HPLC grade acetonitrile/10% ultra-pure water containing 1 mg / mL of BHT. Samples were separated on a Thermo-Finnigan Surveyor HPLC system, using a Thermo

BetaBasic-8 column (100 x 4.6 mm, 5 μm) with a Securityguard pre-column containing a C_{18} cartridge (various other C_{18} reverse-phase and silica columns were also tested but the BetaBasic-8 column gave consistent and stable retention times and separation). The auto-sampler was set at 10 ^{o}C and the column oven was set at 25 ^{o}C. Flow rate was 1 mL / min. Solvent A was ultra-pure water and solvent B was HPLC-grade acetonitrile. A linear gradient was used: 0 min (20% A, 80% B), 30 min (20% A, 80% B), 35 min (100% B), 40 min (100% B), 45 min (20% A, 80% B). PDA detection was at 270 nm (overall 200-600 nm) followed by fluorescence detection (excitation 294 nm, emission 326 nm). Sample injection volume was 20 μL. Calibration curves were produced with various tocopherols. Inter-day variation of external standards was less than 1%.

6.2.4 Extraction and HPLC Analysis of Pigments (Carotenoids and Chlorophylls)

Triplicate sub-samples (3 x 200 mg) were taken for each sample and transferred into separate 10 mL screw-cap glass test tubes. In a low-light fume-cabinet, to each sub-sample, 5 mL of 1:1 n-hexane:diethyl ether was added followed by 25 μL of APO (trans-β-apo-8′-carotenal; 170 mg / L in 100% ethanol). Each tube was vortex mixed for 1 minute and the sub-samples left for 20 minutes at 20 ^{o}C, with vortex mixing every 5 minutes to ensure optimal extraction. The sub-samples were centrifuged (Kubota centrifuge, 4,000 rpm, 20 ^{o}C, 10 minutes) and the supernatant of each sub-sample transferred to individual 50 mL round-bottomed flasks and covered with aluminium foil to prevent photo-degradation of the pigments. Each sub-sample was re-extracted twice with 5 mL 1:1 n-hexane:diethyl ether and the individual supernatants added to the previous supernatants of the corresponding sub-sample; in the second and third extractions no additional APO was added. The pooled supernatants in each round-bottomed flask (approximately 3 x 5 mL for each sub-sample) were evaporated to dryness (water bath set at 40 ^{o}C) and the residues were re-suspended in 1 mL of 1:1 n-hexane:acetone and the solvent transferred to 2 mL screw-top centrifuge tubes for centrifugation to remove any particulate material (Sigma 2-16K centrifuge, 15,493 *g* [13,000 rpm], 4 ^{o}C, 10 minutes). The clarified supernatants were then transferred to 1.5 mL HPLC vials. Samples were always loaded onto the HPLC on the day of extraction and an APO control was always run with the samples. HPLC was performed using a similar system to that of Mendes-Pinto *et al*. (2005), but with minor modifications. The HPLC system used was a Thermo-Finnigan "Surveyor" system with a quaternary pump, solvent de-gasser, a thermostatically-controlled auto-sampler (set at 10^{o}C) and

a thermostatically-controlled column oven (set at 25 ^{o}C) and a photo-diode array detector. The column used was a Phenomenex Luna C_{18} (2) (250 x 4.6 mm, 5 μm) with a "Securityguard" pre-column containing a C_{18} cartridge. Solvent A was 90% HPLC grade acetonitrile with 10% ultra-pure water, and solvent B was high grade ethyl acetate. The flow rate was 1 mL / minute. The linear gradient used was a minor modification of one previously developed (Mendes-Pinto *et al*., 2005): 0 minutes (100% A), 31 minutes (40% A, 60% B), 40 minutes (40% A, 60% B), 43 minutes (100% B), 48 minutes (100% B), 52 minutes (100% A), 60 minutes (100% A). Photo-diode array data was collected at 440 nm (carotenoids and APO) and 650 nm (chlorophylls [free and esterified] and related pigments [pheophytins and pheophorbides]) and overall data between 200-800 nm. Different concentrations of a combined standard mixture of lutein, chlorophyll a and b, lycopene and β-carotene were injected to produce the pigment calibration curves.

6.2.5 Extraction and Quantification of Total Phenolics and Total Condensed Tannins

Initial phenolic studies were performed on **s**ubsamples of the freeze-dried chestnut pericarp and integument samples (3 x 40 mg) by extraction with 1 mL of 70% methanol at 70 °C for 60 minutes in 2 mL screw-top microtubes, using a vortex mixer every 5 minutes to optimize extraction. The samples were centrifuged (17,000*g*, 4 °C, 20 minutes), and subsamples of the supernatant (3 x 10 μL) were used for determining total phenolics contents following the Folin-Ciocalteau spectrophotometer method (Javanmardi *et al*., 2003). Gallic acid was used to produce the calibration curve and results were expressed as mg gallic acid equivalents (GAE) / g fresh weight. Total condensed tannins were determined using the acid/1-butanol method previously described (Huemmer *et al*., 2008). Essentially 75 μL of each extract replicate was added to 1.4 mL of HCl:1-butanol (5:95 v/v) in a screw-cap 2 mL micro-tube, sealed, and heated at 95 ^{o}C for 2 hours. The samples were centrifuged (Sigma 2-16K centrifuge, 15,493 *g* [13,000 rpm], 4 ^{o}C, 10 minutes) and the absorbances of the supernatants were measured at 555 nm. A calibration curve was produced using a partially-purified condensed tannin extract that was prepared from the integument of cultivar Longal.

6.2.6 Preparation of Chestnut Integument Condensed Tannin Standard

Integuments (5 g of each triplicate) of cultivar Longal were mixed and from this mixture sub-samples (6 x 200 mg) were weighed into 10 mL screw-cap glass tubes. To each tube 5 mL of 70% methanol was added, the tubes sealed, and extracted at 70 ^{o}C for 1 hour with vortex mixing

every 10 minutes to optimize extraction. The tubes were centrifuged (4,000 rpm, 20 ^{o}C, 10 minutes) and the supernatants pooled. The pooled supernatant sample was evaporated (water bath set at 40 ^{o}C) to a minimum volume of approximately 3mL. A silica solid-phase extraction column (2 cm diameter, 10 cm height) was sequentially pre-washed with 40 mL 100% methanol followed by 80 mL ultra-pure water. The sample was loaded onto the column using a Vac-elut SPE system. The unbound (loading) and first elution (20 mL of 20% methanol / 80% dichloromethane) were discarded and the condensed tannins were eluted with 20 mL of 100% methanol. The condensed tannin fraction was transferred to a pre-weighed 50 mL glass beaker and the solvent removed using a stream of N_2 gas. The beaker was re-weighed and the dry material re-suspended in 100% methanol to give a 5 mg / mL stock solution which was then used for producing the condensed tannin calibration curve.

6.2.7 Initial HPLC Analyses of Phenolics

Subsamples of the freeze-dried chestnut pericarp and integument samples (3 x 40 mg) were extracted, as described above, and HPLC analyses were performed using methods previously described (Bennett *et al*., 2006; De Vasconcelos *et al*., 2007). Essentially the previously reported multi-purpose reverse-phase method was used; a Phenomenex Luna C18 (*2*) (250 mm x 4.6 mm, 5 µm) main column, with a Securityguard pre-column (Phenomenex, United Kingdom) and a C_{18} cartridge was used 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.

6.2.8 Solvent Extraction Studies

As part of the evaluation of the efficacy of the extraction procedure, different solvents were tested to determine their efficiencies, focusing on the phenolics (totals and selected HPLC evaluations). Triplicate samples (3 x 40 mg) of pericarp and integument were extracted with 1 mL of the specified solvents in 2 mL screw-cap micro-tubes, and for the 70 ^{o}C extractions a heating block was used. The following extraction solvents and temperatures were tested: water at 20 and 70 ^{o}C, 70% v/v methanol / 30% water at 20 and 70 ^{o}C, 70% v/v ethanol/30% water at 20 and 70 ^{o}C, 70% v/v acetone / 30% water at 20 ^{o}C and 100% methylethyl ketone at 20 ^{o}C. For the

20 ^{o}C extractions each one of these was performed for 48 hours and for the 70 ^{o}C extractions for 1 hour. After extraction the tubes were centrifuged (13,000 rpm, 4 ^{o}C, 20 minutes), and the supernatant of each sub-sample transferred to new 2 mL screw-cap tubes. For HPLC profile comparisons of the different solvent extracts, the cultivar Longal was chosen; this is one of the cultivars most widely used in Portugal and thus generates large percentage of waste materials. Sub-samples (10g) of Longal pericarp from stages A and B were combined and the same combination was done for the integument tissues. The water, methanol, ethanol and acetone extracts were analyzed directly by HPLC; 150 µL sub-samples from each replicate, for each Longal sample, were pooled in single HPLC vials. For all the solvent extracts and for the two tissues, sub-samples were taken to determine total phenolics and total procyanidins using the previously described methods.

6.2.9 Statistical Analyses

Data was analyzed by one way ANOVA followed by Duncan's new multiple range test with a 0.05 significance level, using the *SuperANOVA* (1.1 version, Abacus Concepts Inc., 1989) statistical package.

6.3 Results and Discussion

6.3.1 Vitamin E and Pigments Contents

Neither tocopherols nor carotenoids/chlorophylls were detected in any of the pericarp or integument samples; these compounds were below the detection limits. Recovery of spiked (tocopherol) and internal standard (APO for pigments) were >95% in both extractions (data not shown), indicating that the absence of these metabolites was not due to poor extraction efficiencies.

6.3.2 Total Phenolics in 70% Methanol Extracts

Initial studies on total phenolics variation in pericarp and integument revealed the highest values in the integument, with exception to Longal A. The integument of Longal cultivar in stage B showed the highest value (126.75 mg g^{-1} FW), while the lowest value was found in the pericarp of Lada cultivar stage B (58.41 mg g^{-1} FW) (Table 6.2). The content of total phenolics in the pericarp of Martaínha cultivar was significantly different between the processing stages A and B, while the values found in the integument were more significantly different between stages

in Longal and Judia cultivars (Table 6.2). These values are higher when compared with the ones presented by Vázquez *et al*. (2008) (36.0 mg g^{-1} FW) for the whole chestnut shell (integument and pericarp) extracted with 80% methanol at boiling point. The present values are also higher than the total phenolic content found in another work that revealed low values in the pericarp (0.4 to 2.3 mg g^{-1} DM; moisture content was not determined) and integument (1.9 to 8.2 mg g^{-1} DM) extracted with 50% methanol at room temperature in an ultrasound bath (Živković *et al*., 2008).

6.3.3 Gallic Acid in 70% Methanol Extracts

The contents of gallic acid found were higher in the integument of the Lada cultivar stage B (0.35 mg g^{-1} FW) and in the pericarp of the Judia cultivar stage B (0.36 mg g^{-1} FW). The lower values of gallic acid were found in the pericarp of the cultivars Longal B and Judia A (0.14 mg g^{-1} FW) (Table 6.2). The gallic acid values reveal that, in both pericarp and integument, the differences between the processing stages A and B were significantly different only in the cultivars Longal and Judia.

6.3.4 Ellagic Acid in 70% Methanol Extracts

Compared with integument, the pericarp of the cultivars analysed had higher contents of ellagic acid. The highest values were present in the pericarp of the cultivar Longal B (0.19 mg g^{-1} FW). However, the values found in the pericarp of the four Portuguese cultivars were not significantly different between stages or cultivars. The lowest values of ellagic acid were present in the integument of the cultivars Longal B, Judia A and Lada A (0.03 mg g^{-1} FW). The ellagic acid contents found in the chestnut integument were more significantly different between the processing stages A and B of the cultivar Judia (Table 6.2). These values are within the range previously presented by Vekiari *et al*. (2008) that also showed that the content of ellagic acid was higher in the pericarp tissue for un-hydrolysed samples. On the other hand, the same study revealed higher values for the hydrolysed samples in the pericarp (0.7 to 6.0 mg g^{-1} FW) and integument (0.5 to 0.8 mg g^{-1} FW) tissues when comparing with the values in the present work. These higher values observed post-hydrolysis are due to the release of ellagic acid from ellagitannins such as vescalagin and castalagin.

6.3.5 Vescalagin and Castalagin in 70% Methanol Extracts

Vescalagin and mainly castalagin were higher in the pericarp tissue of the chestnut samples studied (Table 6.2). The highest values of vescalagin and castalagin were found in the pericarp of the cultivar Martaínha B (0.13 and 0.85 mg g^{-1} FW, respectively). The vescalagin contents were lower in the integument of the cultivar Judia A (0.04 mg g^{-1} FW), while the castalagin contents were lower in the integument of the cultivars Longal B, Judia A and Lada (0.07 mg g^{-1} FW). The values of vescalagin found in the pericarp were not significantly different between cultivars or stages, but for the integument values the difference between the stages A and B was only significant in cultivar Judia. The castalagin contents found in the studied samples were significantly different only between the processing stages of Longal and Judia in the integument values.

6.3.6 Acutissimins, Unidentified Tannins and the Ellagic Acid Derivative in 70% Methanol Extracts

Tissues also contained two tannins (T1 and T2; provisionally identified from their UV-visible spectra which were nearly identical to the ellagitannin standards), acutissimins A and B, and an unidentified ellagic acid derivative (UEA) (Table 6.2). The pericarp of the analysed samples presented higher values of acutissimin B, while the other tannins presented lower and very similar contents. Only acutissimin A revealed significant differences between cultivars in the pericarp, while the same tannin was the only one with no significant differences in the integument (Table 6.2). The UEA derivative revealed higher values in the pericarp and very low values in the integument, although the differences between the cultivars or the stages in the pericarp were not significant (Table 6.2).

6.3.7 Total Phenolics in Different Solvent Extracts

The total phenolic content revealed that the most efficient extraction solvent was 70% aqueous acetone (20 ºC), with higher values for the integument tissues with exception of Longal A. For this solvent, the cultivar Longal B in the integument presented the highest value of total phenolics (136.35 mg 100^{-1}g FW), and the cultivar Lada B in the pericarp presented the lowest (68.51 mg g^{-1} FW). On the other hand methylethylketone (MEK) was the least efficient extraction solvent tested, with the highest value in the integument of Longal B (6.62 mg g^{-1} FW) and the lowest in the pericarp of Lada B (2.22 mg g^{-1} FW). After 70 % acetone, the extraction efficiencies in order were 70% methanol (MeOH) at 70 ºC > 70% MeOH at 20 ºC > 70% ethanol

(EtOH) at 70 ºC > 70% EtOH at 20 ºC > water at 70 ºC > water at 20 ºC (Tables 6.2 and 6.3). All the extraction solvents revealed (with exception to Longal A) higher contents of total phenolics in the integument tissues, in which the cultivar Longal B presented the highest values of these compounds, with the exception of the extraction solvent water at 20 ºC. The temperature had more of a positive effect in the pericarp extraction (Table 6.3). A previous study with different extracting solvents at boiling point revealed a higher efficiency of 2.5% Na_2SO_3 aqueous in the extraction of total phenolics in chestnut shell (134.0 mg g^{-1} FW), while the 100% ethanol showed the lowest extraction efficiency (6.0 mg g^{-1} FW) (Vázquez *et al*., 2008). In a second study by the same authors a high extraction efficiency was found with 2.5% Na_2SO_3 + NaOH at 90 ºC for total phenolics in chestnut shell (188.4 mg g^{-1} FW), while water at 70 ºC had the lower extraction efficiency (42.0 mg g^{-1} FW) (Vázquez *et al*., 2009). The values of total phenolics obtained with water extraction at 70 ºC in the present work were similar to the ones found by Barreira *et al*. (2008) with boiling water in the pericarp tissue (25.4 mg g^{-1} FW). However, in this previous work the values found in the integument (102.6 mg g^{-1} FW) were higher than the ones revealed in the present study. Studies have been done on the total phenolics contents in almonds - in the blanching water (0.5 to 1.5 mg GAE g^{-1} FW) and then obtaining the skins that were then extracted with HCl:H_2O:methanol (3.7:46.3:50, v/v/v) during 16 hours at 4°C (0.1 to 0.3 mg GAE g^{-1} FW) (Milbury *et al*., 2006). Other work analyzed the total phenolic contents of peanut shells obtaining values of 118, 112 and 81 mg g^{-1} DM, extracted with 30.8% aqueous ethanol (30.9ºC, for 12.2 min), 63.8% aqueous methanol (57.3ºC, for 21.6 min) and 100% water (50.4ºC, for 10.1 min), respectively (Ballard *et al*., 2009). Alasalvar *et al*. (2009) determined the content of total phenolics in hazelnut skins with 80% aqueous acetone (686 mg catechin equivalents g^{-1} FW) and 80% aqueous methanol (701 mg catechin equivalents g^{-1} FW) at 50ºC, for 30 min. Overall, the values of total phenolics obtained in the present work are higher that the contents found in almonds and closer to the ones found in peanut shells, with hazelnuts presenting the highest total phenolic contents of the referred nuts.

6.3.8 Total Condensed Tannins in Different Solvent Extracts

The most efficient solvent in the extraction of condensed tannins was 70% acetone (20 ºC), showing much higher values in the integument tissue. For this solvent, the cultivar Longal B in the integument presented the highest value of condensed tannins (110.35 mg g^{-1} FW), and the cultivar Lada B in the pericarp presented the lowest (16.32 mg g^{-1} FW). The water was the least efficient solvent, with the highest value in the integument of Martaínha A (30.94 mg g^{-1} FW at

20 ºC) and the lowest in the pericarp of Lada B (3.14 mg g^{-1} FW at 20 ºC) (Table 6.4). The efficiency of extraction for the different solvents was similar to that of the total phenolics (Tables 6.2 and 6.3). Once again, the extraction solvents revealed higher contents of condensed tannins in the integument tissue and the temperature had more of a positive effect in the pericarp extractions for the majority of the samples.

6.3.9 HPLC of Different Extracts of Longal Pericarp and Integument - Combined Stages A and B

The HPLC analyses showed a complex phenolic profile in both pericarp and integument samples; example chromatograms of the various standards and a 70% aqueous acetone extract of Longal pericarp are shown in Figure 6.2.

The values of gallic acid found were not significantly different between the extraction solvents either in the pericarp or integument tissue. However, the highest values of this compound were obtained using the 70% acetone in the pericarp and integument (1.07 and 1.05 mg g^{-1} FW, respectively) (Table 6.5). The lowest values were obtained with water at 70 ºC (0.64 mg g^{-1} FW) for the pericarp and with 70% MeOH at 70 ºC and 20% EtOH at 20 ºC for integument (0.75 mg g^{-1} FW).

The solvent 70% acetone at 20 ºC was the most efficient in the extraction of ellagic acid in the pericarp (0.30 mg g^{-1} FW), while the extraction with 70% ethanol at 20 ºC obtained the highest values of this compound in the integument (0.22 mg g^{-1} FW). The less efficient solvent was water at 70 ºC (0.02 and 0.03 mg g^{-1} FW for pericarp and integument, respectively). The values of ellagic acid found in the pericarp were significantly different between the solvents, while the differences among the values obtained in the integument were less significant (Table 6.5).

The castalagin values obtained in the pericarp and integument were higher than the values of vescalagin. The castalagin and vescalagin contents were higher in the pericarp when extracted with 70% acetone (0.59 and 0.26 mg g^{-1} FW, respectively). On the other hand, the values from the integument tissue revealed that the higher values of castalagin were obtained with water at 70 ºC and 70% acetone (0.33 mg g^{-1} FW), while the higher values of vescalagin were obtained with water at 20 ºC (0.14 mg g^{-1} FW). The lower values of castalagin in the pericarp were obtained with 70% methanol at 70 ºC (0.21 mg g^{-1} FW), and in the integument with 70% ethanol at 20 ºC (0.19 mg g^{-1} FW).

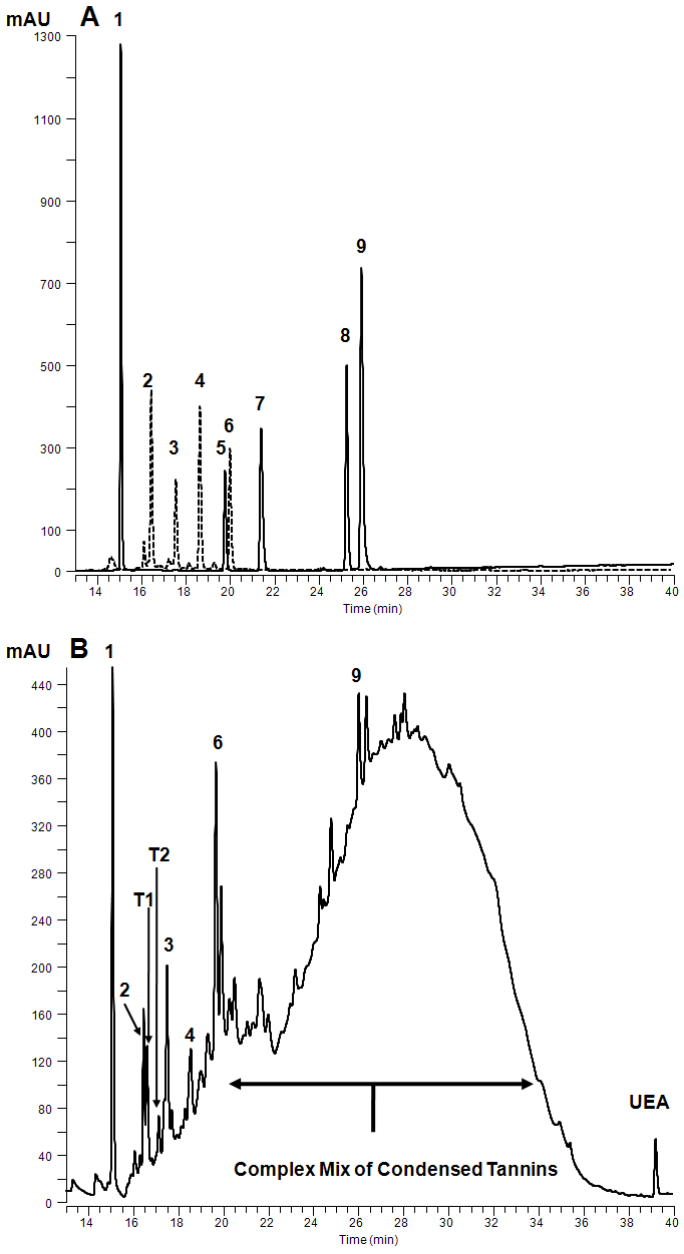

Figure 6.2 - Example chromatograms of combined standards (A) and a 70% acetone extract of Longal pericarp (B).

Combined standards (A, over-laid chromatograms - solid line = phenolic standards [5µL of 0,1 mg/mL each phenolic] and dashed line = ellagitannin standards [5µL of 0,1mg / mL each tannin) and a 70% acetone extract of Longal pericarp (B - 5µL). Peak identification confirmed from retention time and spectral comparisons with standards: 1 = gallic Acid, 2 = vescalagin, 3 = castalagin, 4 = acutissimin A, 5 = chlorogenic acid, 6 = acutissimin B, 7 = epicatechin, 8 = rutin (quercetin 3-*O*-rhamnoglucoside), 9 = ellagic Acid. T1 and T2 = unidentified hydrolyzable tannins (based on similarity to UV-visible spectra of ellagitannin standards), UEA = unidentified ellagic acid derivative (based on similarity to UV-visible spectra of ellagic acid standard). Peaks not labelled in the sample chromatogram were not identified in the present study.

The values of vescalagin were found to be lower in the pericarp when extracted with 70% methanol at 70 ºC (0.06 mg g^{-1} FW), and lower in the integument with 70% ethanol at 20 ºC and 70 ºC (0.06 and 0.05 mg g^{-1} FW, respectively). Neither of these two compounds revealed significant differences between the extraction solvents used in the pericarp or the integument. The highest values of tannins T1 and T2, acutissimins A and B, and UEA were obtained using 70% acetone in the pericarp. From these tannins, acutissimin B had the highest values in the pericarp tissue with 70% acetone (0.84 mg g^{-1} FW), while the highest value of acutissimin B was obtained with 70% ethanol at 20 ºC in the integument (0.24 mg g^{-1} FW). The tannin T2 revealed the lowest values in the pericarp with 70 % methanol at 20 ºC (0.02 mg g^{-1} FW), and in the integument with water at 20 ºC (0.03 mg g^{-1} FW). The UEA values were low in the pericarp tissue, and were much lower in the integument tissue, where this compound was below the detection limit when water was used as the extraction solvent. The highest value of this compound was found in the pericarp with 70% acetone (0.17 mg g^{-1} FW). The values of T2 and acutissimin A in the pericarp were not significantly different between the solvents used, while in the integument neither the tannins T1 and T2, acutissimins A and B, nor UEA revealed statistically significant differences (Table 6.5).

6.4 Conclusions

This is the first report of acutissimins A and B in chestnut pericarp and integument tissues. The contents of total phenolics and gallic acid in the samples extracted with 70% aqueous methanol were higher in the integument tissue, while the other low molecular weight phenolics presented higher contents in the pericarp. The most efficient extraction solvent for the total phenolics, condensed tannins and low molecular weight phenolics (in Longal) was 70% acetone at 20 ºC. MEK was the least efficient extraction solvent for total phenolics. The integument had the highest values of total phenolics and condensed tannins. The pericarp and integument samples of the cultivar Longal were richer in gallic acid and castalagin, while UEA revealed the lowest values. The differences between the values obtained with different solvents, in the cultivar Longal, were not significant for any of the low molecular weight phenolics in the integument. However, in the pericarp, the values found were significantly different between the extraction solvents for the ellagic acid, tannin T1 and acutissimin B, and UEA. Although neither tocopherols nor carotenoids/chlorophylls were detected, the samples of chestnut pericarp and integument analysed had significant values of phenolics. It is clear from this and other studies that these chestnut tissues can be used as a source of valuable phenolics, but further studies need

to be performed to optimise the extraction efficiencies with the most effective and lowest cost solvent and to have complete LC-MS identification of the different phenolics including full characterisation of the condensed tannins in these tissues.

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Table 6.2 - Initial studies on variation in total phenolics and specific phenolics in extracts of pericarp and integument of the four cultivars prepared using 70% v/v methanol at 70 °C, from the two processing stages (A - fresh; B - after storage).

CV	Tissue	Total Phenolics [1] (n=3)	Gallic Acid (n=3)	Vescalagin (n=3)	Castalagin (n=3)	Ellagic Acid (n=3)	Tannin T1 (n=3)	Tannin T2 (n=3)	Acutissimin A (n=3)	Acutissimin B (n=3)	UEA (n=3)
LG A	Per.	84.87 ± 3.79 ^{d}	0.33 ± 0.05 ^{bc}	0.07 ± 0.03 ^{a}	0.57 ± 0.29 ^{ab}	0.14 ± 0.05	0.08 ± 0.04	0.08 ± 0.03 ^{ab}	0.07 ± 0.01 ^{b}	0.48 ± 0.21	0.11 ± 0.05
LG B	Per.	79.77 ± 1.78 ^{cd}	0.14 ± 0.04 ^{a}	0.05 ± 0.03 ^{a}	0.39 ± 0.07 ^{a}	0.19 ± 0.03	0.11 ± 0.04	0.09 ± 0.02 ^{b}	0.08 ± 0.02 ^{b}	0.43 ± 0.17	0.14 ± 0.03
JD A	Per.	61.92 ± 4.97 ^{a}	0.14 ± 0.03 ^{a}	0.06 ± 0.03 ^{a}	0.41 ± 0.07 ^{a}	0.18 ± 0.03	0.09 ± 0.04	0.09 ± 0.02 ^{b}	0.08 ± 0.01 ^{b}	0.42 ± 0.15	0.13 ± 0.03
JD B	Per.	65.61 ± 6.45 ^{ab}	0.36 ± 0.06 ^{c}	0.09 ± 0.05 ^{ab}	0.58 ± 0.27 ^{ab}	0.16 ± 0.05	0.10 ± 0.06	0.09 ± 0.03 ^{ab}	0.08 ± 0.01 ^{b}	0.52 ± 0.23	0.12 ± 0.05
MRT A	Per.	72.92 ± 4.67 ^{bc}	0.27 ± 0.02 ^{b}	0.10 ± 0.03 ^{ab}	0.82 ± 0.15 ^{b}	0.16 ± 0.01	0.09 ± 0.02	0.09 ± 0.01 ^{ab}	0.08 ± 0.01 ^{b}	0.52 ± 0.02	0.16 ± 0.01
MRT B	Per.	62.28 ± 7.54 ^{a}	0.27 ± 0.02 ^{b}	0.13 ± 0.02 ^{b}	0.85 ± 0.18 ^{b}	0.17 ± 0.01	0.08 ± 0.03	0.09 ± 0.02 ^{ab}	0.08 ± 0.01 ^{b}	0.51 ± 0.07	0.16 ± 0.02
LD A	Per.	62.05 ± 4.51 ^{a}	0.17 ± 0.01 ^{a}	0.06 ± 0.02 ^{a}	0.61 ± 0.12 ^{ab}	0.16 ± 0.02	0.06 ± 0.02	0.05 ± 0.02 ^{a}	0.05 ± 0.01 ^{a}	0.41 ± 0.06	0.15 ± 0.02
LD B	Per.	58.41 ± 4.45 ^{a}	0.17 ± 0.01 ^{a}	0.07 ± 0.01 ^{a}	0.62 ± 0.10 ^{ab}	0.17 ± 0.01	0.07 ± 0.03	0.06 ± 0.01 ^{ab}	0.05 ± 0.01 ^{a}	0.42 ± 0.03	0.16 ± 0.01
Sig. Level		P=0.0001 ***	P=0.0001 ***	P=0.0679 ns	P=0.0492 *	P=0.4660 ns	P=0.6816 ns	P=0.1197 ns	P=0.0055 **	P=0.9164 ns	P=0.3774 ns
LG A	Int.	76.04 ± 8.93 ^{a}	0.30 ± 0.05 ^{b}	0.07 ± 0.03 ^{bc}	0.21 ± 0.08 ^{b}	0.05 ± 0.02 ^{ab}	0.05 ± 0.01 ^{abc}	0.04 ± 0.01 ^{ab}	0.04 ± 0.02	0.07 ± 0.02 ^{abc}	0.01 ± 0.00 ^{a}
LG B	Int.	126.75 ± 9.27 ^{c}	0.24 ± 0.03 ^{a}	0.05 ± 0.01 ^{ab}	0.07 ± 0.00 ^{a}	0.03 ± 0.00 ^{a}	0.03 ± 0.01 ^{a}	0.03 ± 0.01 ^{a}	0.04 ± 0.01	0.05 ± 0.01 ^{a}	0.00 ± 0.00 ^{a}
JD A	Int.	82.96 ± 5.74 ^{a}	0.22 ± 0.03 ^{a}	0.04 ± 0.00 ^{a}	0.07 ± 0.00 ^{a}	0.03 ± 0.00 ^{a}	0.03 ± 0.01 ^{a}	0.03 ± 0.01 ^{a}	0.03 ± 0.01	0.04 ± 0.01 ^{a}	0.00 ± 0.00 ^{a}
JD B	Int.	103.41 ± 4.79 ^{b}	0.32 ± 0.04 ^{b}	0.08 ± 0.01 ^{c}	0.21 ± 0.08 ^{b}	0.06 ± 0.03 ^{b}	0.06 ± 0.02 ^{abc}	0.05 ± 0.01 ^{bc}	0.05 ± 0.01	0.07 ± 0.02 ^{abc}	0.01 ± 0.00 ^{a}
MRT A	Int.	105.98 ± 4.96 ^{b}	0.32 ± 0.01 ^{b}	0.07 ± 0.01 ^{bc}	0.10 ± 0.01 ^{a}	0.07 ± 0.01 ^{b}	0.07 ± 0.04 ^{bc}	0.04 ± 0.00 ^{ab}	0.04 ± 0.01	0.09 ± 0.01 ^{c}	0.01 ± 0.00 ^{bc}
MRT B	Int.	105.20 ± 16.93 ^{b}	0.33 ± 0.04 ^{b}	0.08 ± 0.01 ^{c}	0.10 ± 0.01 ^{a}	0.07 ± 0.03 ^{b}	0.08 ± 0.03 ^{c}	0.06 ± 0.01 ^{c}	0.05 ± 0.02	0.08 ± 0.02 ^{bc}	0.01 ± 0.01 ^{c}
LD A	Int.	90.52 ± 4.52 ^{ab}	0.34 ± 0.04 ^{b}	0.06 ± 0.02 ^{abc}	0.07 ± 0.01 ^{a}	0.03 ± 0.00 ^{a}	0.04 ± 0.00 ^{ab}	0.04 ± 0.00 ^{ab}	0.04 ± 0.01	0.05 ± 0.01 ^{a}	0.01 ± 0.00 ^{ab}
LD B	Int.	104.36 ± 7.20 ^{b}	0.35 ± 0.02 ^{b}	0.06 ± 0.02 ^{abc}	0.07 ± 0.00 ^{a}	0.04 ± 0.00 ^{ab}	0.05 ± 0.00 ^{abc}	0.04 ± 0.00 ^{ab}	0.04 ± 0.01	0.06 ± 0.00 ^{ab}	0.01 ± 0.00 ^{ab}
Sig. Level		P=0.0001 ***	P=0.0008 ***	P=0.0193 *	P=0.0008 ***	P=0.0102 *	P=0.0379 *	P=0.0032 **	P=0.4844 ns	P=0.0051 **	P=0.0061 **

[1] Data are presented as mean ± SD mg gallic acid equivalents / g Fresh Weight (FW); Data for the specific phenolics are presented as mean ± SD mg phenolic / g FW.

CV - Cultivar; LG - Longal; JD - Judia; MRT - Martaínha; LD - Lada; Per. - Pericarp; Int. - Integument; UEA - Unidentified ellagic acid derivative.

Within each column and for each one of the tissues considered, means with a different letter are significantly different (P<0.05), according to Duncan New Multiple Range Test. NS, not significant (P > 0.05); *, **, and ***, significant at P < 0.05, P < 0.01, and P < 0.001, respectively.

Table 6.3 - Total phenolics in different solvent extracts of pericarp and integument of the four chestnut cultivars, from the two processing stages (A - fresh; B - after storage).

		100% Water		70% MeOH	70% EtOH		70% Acetone	100% MEK
CV	**Tissue**	**20°C** (n=3)	**70°C** (n=3)	**20°C** (n=3)	**20°C** (n=3)	**70°C** (n=3)	**20°C** (n=3)	**20°C** (n=3)
LG A	Per.	15.36 ± 2.37 ^{c}	26.02 ± 2.01 ^{e}	70.07 ± 2.65 ^{d}	48.67 ± 0.67 ^{c}	61.03 ± 1.91 ^{e}	105.66 ± 1.26 ^{c}	3.25 ± 0.52 ^{c}
LG B	Per.	11.92 ± 0.48 ^{ab}	22.13 ± 1.36 ^{cd}	67.28 ± 0.89 ^{d}	45.79 ± 4.57 ^{c}	51.60 ± 7.42 ^{d}	90.19 ± 6.46 ^{b}	2.92 ± 0.09 ^{bc}
JD A	Per.	14.36 ± 1.48 ^{bc}	24.46 ± 3.35 ^{de}	57.95 ± 2.64 ^{c}	38.30 ± 5.26 ^{b}	51.29 ± 3.59 ^{d}	92.30 ± 7.28 ^{b}	2.96 ± 0.51 ^{bc}
JD B	Per.	11.35 ± 0.70 ^{a}	19.00 ± 0.63 ^{bc}	46.15 ± 2.92 ^{a}	32.24 ± 1.56 ^{ab}	42.01 ± 2.12 ^{bc}	75.38 ± 3.00 ^{a}	2.74 ± 0.31 ^{abc}
MRT A	Per.	18.40 ± 0.95 ^{d}	26.30 ± 0.97 ^{e}	55.39 ± 4.06 ^{bc}	50.91 ± 3.61 ^{c}	56.18 ± 1.78 ^{de}	95.01 ± 7.02 ^{b}	2.53 ± 0.16 ^{ab}
MRT B	Per.	11.70 ± 2.44 ^{a}	13.75 ± 2.57 ^{a}	47.24 ± 4.45 ^{a}	37.93 ± 3.50 ^{b}	35.38 ± 4.27 ^{ab}	78.15 ± 6.21 ^{a}	2.24 ± 0.10 ^{a}
LD A	Per.	13.00 ± 0.39 ^{abc}	24.40 ± 1.65 ^{de}	48.50 ± 8.64 ^{ab}	37.56 ± 2.35 ^{b}	48.63 ± 6.14 ^{cd}	89.96 ± 8.03 ^{b}	2.49 ± 0.14 ^{ab}
LD B	Per.	10.90 ± 0.56 ^{a}	18.53 ± 1.21 ^{b}	45.31 ± 2.97 ^{a}	30.85 ± 3.92 ^{a}	34.16 ± 2.85 ^{a}	68.51 ± 5.27 ^{a}	2.22 ± 0.17 ^{a}
Sig. level		P=0.0001 ***	P=0.0001 ***	P=0.0001 ***	P=0.0001 ***	P=0.0001 ***	P=0.0001 ***	P=0.0065 **
LG A	Int.	23.49 ± 3.59 ^{a}	15.06 ± 1.67 ^{a}	66.84 ± 7.70 ^{a}	56.92 ± 2.37 ^{a}	58.94 ± 6.87 ^{a}	91.02 ± 6.05 ^{a}	4.87 ± 0.90 ^{bc}
LG B	Int.	52.71 ± 9.92 ^{b}	52.11 ± 1.79 ^{e}	104.41 ± 2.13 ^{d}	77.88 ± 11.13 ^{d}	82.00 ± 6.18 ^{c}	136.35 ± 15.76 ^{e}	6.62 ± 0.90 ^{d}
JD A	Int.	26.59 ± 4.02 ^{a}	17.21 ± 1.83 ^{a}	76.00 ± 4.72 ^{ab}	58.55 ± 4.98 ^{ab}	61.71 ± 1.97 ^{ab}	95.98 ± 10.41 ^{ab}	3.37 ± 0.17 ^{a}
JD B	Int.	55.69 ± 9.37 ^{bc}	35.06 ± 8.42 ^{c}	83.40 ± 5.14 ^{bc}	73.20 ± 2.65 ^{cd}	70.59 ± 4.63 ^{b}	120.85 ± 1.22 ^{cd}	4.50 ± 0.64 ^{abc}
MRT A	Int.	65.82 ± 1.84 ^{c}	24.12 ± 1.12 ^{b}	102.13 ± 4.49 ^{d}	78.32 ± 4.06 ^{d}	82.68 ± 8.63 ^{c}	124.36 ± 7.98 ^{de}	5.61 ± 0.80 ^{cd}
MRT B	Int.	55.10 ± 8.81 ^{bc}	44.42 ± 3.93 ^{d}	91.12 ± 8.00 ^{c}	67.20 ± 9.82 ^{abcd}	70.53 ± 6.16 ^{b}	128.94 ± 1.12 ^{de}	4.98 ± 0.31 ^{bc}
LD A	Int.	27.69 ± 1.08 ^{a}	16.91 ± 1.69 ^{a}	77.25 ± 3.69 ^{b}	64.09 ± 5.40 ^{abc}	61.90 ± 5.66 ^{ab}	107.88 ± 8.84 ^{bc}	4.29 ± 0.65 ^{ab}
LD B	Int.	50.74 ± 7.76 ^{b}	43.74 ± 2.54 ^{d}	87.77 ± 6.24 ^{c}	70.27 ± 6.24 ^{bcd}	63.80 ± 2.82 ^{ab}	117.94 ± 3.60 ^{cd}	5.28 ± 0.64 ^{bc}
Sig. level		P=0.0001 ***	P=0.0001 ***	P=0.0001 ***	P=0.0053 **	P=0.0004 ***	P=0.0001 ***	P=0.0013 **

Data are presented as mean ± SD mg gallic acid equivalents / g FW.

CV - Cultivar; LG - Longal; JD - Judia; MRT - Martaínha; LD - Lada; Per. - Pericarp; Int. - Integument; MeOH - Methanol; EtOH - Ethanol; MEK - Methylethylketone. Within each column and for each one of the tissues considered, means with a different letter are significantly different ($P<0.05$), according to Duncan New Multiple Range Test. ** and ***, significant at $P < 0.01$ and $P < 0.001$, respectively.

Table 6.4 - Total condensed tannins in different extracts of pericarp and integument of the four chestnut cultivars, from the two processing stages (A - fresh; B - after storage).

		100% Water		70% MeOH		70% EtOH		70% Acetone
CV	**Tissue**	**20°C** (n=3)	**70°C** (n=3)	**20°C** (n=3)	**70°C** (n=3)	**20°C** (n=3)	**70°C** (n=3)	**20°C** (n=3)
LG A	Per.	6.49 ± 0.81 ^{e}	5.64 ± 0.75 ^{bc}	11.55 ± 0.35 ^{c}	12.64 ± 0.84 ^{e}	10.03 ± 5.51 ^{c}	9.05 ± 0.30 ^{c}	35.11 ± 1.87 ^{d}
LG B	Per.	3.59 ± 0.79 ^{ab}	5.93 ± 0.40 ^{bc}	10.34 ± 0.60 ^{c}	10.90 ± 0.51 ^{d}	8.15 ± 0.57 ^{bc}	10.29 ± 0.47 ^{d}	28.19 ± 3.08 ^{c}
JD A	Per.	5.37 ± 0.73 ^{cde}	4.73 ± 0.27 ^{ab}	8.49 ± 0.98 ^{b}	9.48 ± 1.05 ^{bc}	7.70 ± 3.03 ^{abc}	6.19 ± 0.43 ^{a}	22.54 ± 2.82 ^{b}
JD B	Per.	4.05 ± 0.87 ^{abc}	5.08 ± 0.61 ^{abc}	6.19 ± 0.14 ^{a}	7.66 ± 0.20 ^{a}	4.62 ± 0.18 ^{ab}	8.31 ± 0.52 ^{c}	17.89 ± 1.37 ^{a}
MRT A	Per.	5.70 ± 0.69 ^{de}	6.34 ± 1.78 ^{c}	11.07 ± 1.94 ^{c}	10.49 ± 0.77 ^{cd}	6.52 ± 0.25 ^{abc}	8.74 ± 0.38 ^{c}	29.31 ± 2.46 ^{c}
MRT B	Per.	4.94 ± 0.92 ^{bcd}	4.66 ± 0.37 ^{ab}	7.78 ± 1.42 ^{ab}	8.70 ± 0.48 ^{ab}	6.15 ± 0.42 ^{abc}	7.20 ± 0.77 ^{b}	23.12 ± 2.28 ^{b}
LD A	Per.	4.10 ± 0.53 ^{abc}	4.61 ± 0.99 ^{ab}	7.75 ± 0.71 ^{ab}	10.30 ± 0.45 ^{cd}	4.02 ± 0.25 ^{ab}	8.09 ± 0.90 ^{bc}	27.54 ± 2.28 ^{c}
LD B	Per.	3.14 ± 0.46 ^{a}	4.01 ± 0.19 ^{a}	6.83 ± 0.70 ^{ab}	7.83 ± 0.45 ^{a}	3.72 ± 0.18 ^{a}	6.12 ± 0.06 ^{a}	16.32 ± 0.99 ^{a}
Sig. level		P=0.0006 ***	P=0.0482 *	P=0.0001 ***	P=0.0001 ***	P=0.0368 *	P=0.0001 ***	P=0.0001 ***
LG A	Int.	13.59 ± 2.16 ^{a}	6.28 ± 0.09 ^{a}	26.43 ± 0.35 ^{a}	19.05 ± 0.79 ^{a}	17.00 ± 1.82 ^{a}	26.03 ± 3.13 ^{a}	65.24 ± 2.76 ^{a}
LG B	Int.	9.82 ± 1.57 ^{a}	18.36 ± 1.09 ^{c}	43.18 ± 3.67 ^{c}	27.18 ± 1.15 ^{d}	29.54 ± 4.08 ^{b}	38.53 ± 4.12 ^{c}	110.35 ± 3.06 ^{e}
JD A	Int.	20.74 ± 3.11 ^{b}	8.33 ± 1.36 ^{ab}	33.60 ± 3.03 ^{b}	22.41 ± 0.01 ^{bc}	25.40 ± 2.73 ^{b}	28.31 ± 2.46 ^{ab}	64.14 ± 5.29 ^{a}
JD B	Int.	19.49 ± 0.49 ^{b}	11.76 ± 3.00 ^{b}	42.35 ± 2.46 ^{c}	22.87 ± 2.12 ^{bc}	27.10 ± 1.92 ^{b}	31.71 ± 1.13 ^{b}	101.10 ± 3.54 ^{d}
MRT A	Int.	30.94 ± 1,96 ^{c}	17.10 ± 3.11 ^{c}	40.45 ± 2.91 ^{c}	27.78 ± 1.86 ^{d}	30.42 ± 2.51 ^{b}	38.85 ± 1.06 ^{c}	87.12 ± 3.58 ^{c}
MRT B	Int.	21.50 ± 3.57 ^{b}	22.39 ± 1.56 ^{d}	43.35 ± 6.14 ^{c}	20.78 ± 0.88 ^{ab}	27.27 ± 1.00 ^{b}	30.55 ± 4.21 ^{ab}	97.48 ± 4.72 ^{d}
LD A	Int.	18.33 ± 2.28 ^{b}	10.04 ± 1.76 ^{b}	26.71 ± 1.72 ^{a}	24.05 ± 1.30 ^{c}	25.49 ± 4.08 ^{b}	27.32 ± 3.41 ^{ab}	78.99 ± 4.66 ^{b}
LD B	Int.	21.14 ± 0.56 ^{b}	17.74 ± 1.51 ^{c}	43.69 ± 0.31 ^{c}	20.51 ± 2.55 ^{ab}	27.70 ± 2.33 ^{b}	26.24 ± 1.30 ^{ab}	84.01 ± 4.40 ^{bc}
Sig. level		P=0.0001 ***	P=0.0001 ***	P=0.0001 ***	P=0.0001 ***	P=0.0008 ***	P=0.0001 ***	P=0.0001 ***

Data are presented as mg condensed tannins equivalents / g FW.

CV - Cultivar; LG - Longal; JD - Judia; MRT - Martaínha; LD - Lada; Per. - Pericarp; Int. - Integument; MeOH - Methanol; EtOH - Ethanol.

Within each column and for each one of the tissues considered, means with a different letter are significantly different (P<0.05), according to Duncan New Multiple Range Test. * and ***, significant at P < 0.05 and P < 0.001, respectively.

Table 6.5 - Concentrations of specific phenolics determined by HPLC in different extracts from combined stages A and B of pericarp and integument of the cultivar Longal.

Tissue	Phenolic[a]	100% Water		70% MeOH		70% EtOH		70% Acetone	Signif.
		20°C	70°C	20°C	70°C	20°C	70°C	20°C	level
Per.	**Gallic Acid**	0.77 ± 0.11 $^{c\ 12}$	0.64 ± 0.06 $^{c\ 1}$	0.84 ± 0.11 $^{e\ 12}$	0.81 ± 0.16 $^{b\ 12}$	0.76 ± 0.07 $^{c\ 12}$	0.80 ± 0.10 $^{d\ 12}$	1.07 ± 0.20 $^{d\ 2}$	P=0.1650 ns
	Castalagin	0,34 ± 0.30 ^{b}	0.43 ± 0.24 ^{b}	0.39 ± 0.08 ^{d}	0.21 ± 0.05 ^{a}	0.43 ± 0.39 ^{b}	0.58 ± 0.11 ^{c}	0.59 ± 0.12 ^{bc}	P=0.6281 ns
	Vescalagin	0.14 ± 0.09 $^{ab\ 12}$	0.13 ± 0.04 $^{a\ 12}$	0.15 ± 0.08 $^{ab\ 12}$	0.06 ± 0.03 $^{a\ 1}$	0.07 ± 0.01 $^{a\ 1}$	0.14 ± 0.03 $^{ab\ 12}$	0.26 ± 0.14 $^{ab\ 2}$	P=0.2188 ns
	Ellagic Acid	0.04 ± 0.03 $^{a\ 12}$	0.02 ± 0.01 $^{a\ 1}$	0.22 ± 0.04 $^{bc\ 45}$	0.11 ± 0.06 $^{a\ 23}$	0.17 ± 0.05 $^{ab\ 34}$	0.21 ± 0.02 $^{b\ 4}$	0.30 ± 0.01 $^{ab\ 5}$	P=0.0007 ***
	Tannin T1	0.05 ± 0.05 $^{a\ 1}$	0.09 ± 0.00 $^{a\ 1}$	0.22 ± 0.10 $^{bc\ 2}$	0.03 ± 0.01 $^{a\ 1}$	0.07 ± 0.02 $^{a\ 1}$	0.02 ± 0.01 $^{a\ 1}$	0.27 ± 0.04 $^{ab\ 2}$	P=0.0037 **
	Tannin T2	0.03 ± 0.00 ^{a}	0.07 ± 0.01 ^{a}	0.02 ± 0.03 ^{a}	0.04 ± 0.00 ^{a}	0.06 ± 0.00 ^{a}	0.05 ± 0.01 ^{ab}	0.07 ± 0.09 ^{a}	P=0.7735 ns
	Acutissimin A	0.10 ± 0.03 ^{ab}	0.13 ± 0.03 ^{a}	0.08 ± 0.02 ^{ab}	0.07 ± 0.04 ^{a}	0.08 ± 0.04 ^{a}	0.09 ± 0.04 ^{ab}	0.24 ± 0.16 ^{ab}	P=0.3376 ns
	Acutissimin B	0.15 ± 0.04 $^{ab\ 1}$	0.16 ± 0.08 $^{a\ 1}$	0.32 ± 0.00 $^{cd\ 1}$	0.19 ± 0.16 $^{a\ 1}$	0.21 ± 0.12 $^{ab\ 1}$	0.20 ± 0.12 $^{b\ 1}$	0.84 ± 0.30 $^{cd\ 2}$	P=0.0209 *
	UEA	0.01 ± 0.00 $^{a\ 1}$	0.01 ± 0.00 $^{a\ 1}$	0.09 ± 0.00 $^{ab\ 2}$	0.04 ± 0.04 $^{a\ 12}$	0.05 ± 0.06 $^{a\ 12}$	0.11 ± 0.02 $^{ab\ 23}$	0.17 ± 0.00 $^{a\ 3}$	P=0.0069 **
Sig. level		P=0.0015 **	P=0.0008 ***	P=0.0001 ***	P=0.0001 ***	P=0.0084 **	P=0.0001 ***	P=0.0010 **	
Int.	**Gallic Acid**	0.97 ± 0.38 ^{b}	0.82 ± 0.18 ^{c}	0.94 ± 0.16 ^{c}	0.75 ± 0.17 ^{c}	0.75 ± 0.21 ^{b}	0.79 ± 0.17 ^{c}	1.05 ± 0.24 ^{c}	P=0.7558 ns
	Castalagin	0,22 ± 0.05 ^{a}	0.33 ± 0.01 ^{b}	0.25 ± 0.10 ^{b}	0.21 ± 0.02 ^{b}	0.19 ± 0.08 ^{a}	0.25 ± 0.07 ^{b}	0.33 ± 0.03 ^{b}	P=0.2519 ns
	Vescalagin	0.14 ± 0.14 ^{a}	0.09 ± 0.04 ^{a}	0.09 ± 0.02 ^{a}	0.07 ± 0.04 ^{ab}	0.06 ± 0.01 ^{a}	0.05 ± 0.02 ^{a}	0.12 ± 0.05 ^{ab}	P=0.7901 ns
	Ellagic Acid	0.06 ± 0.04 $^{a\ 12}$	0.03 ± 0.01 $^{a\ 1}$	0.12 ± 0.03 $^{ab\ 123}$	0.12 ± 0.03 $^{ab\ 123}$	0.22 ± 0.01 $^{a\ 3}$	0.15 ± 0.07 $^{ab\ 23}$	0.16 ± 0.09 $^{ab\ 23}$	P=0.0649 ns
	Tannin T1	0.15 ± 0.18 ^{a}	0.04 ± 0.01 ^{a}	0.04 ± 0.01 ^{a}	0.03 ± 0.03 ^{a}	0.06 ± 0.02 ^{a}	0.04 ± 0.00 ^{a}	0.08 ± 0.01 ^{a}	P=0.6373 ns
	Tannin T2	0.03 ± 0.00 ^{a}	0.05 ± 0.02 ^{a}	0.06 ± 0.01 ^{a}	0.04 ± 0.00 ^{a}	0.04 ± 0.00 ^{a}	0.08 ± 0.00 ^{a}	0.05 ± 0.07 ^{a}	P=0.6395 ns
	Acutissimin A	0.04 ± 0.00 ^{a}	0.06 ± 0.03 ^{a}	0.07 ± 0.03 ^{a}	0.05 ± 0.00 ^{a}	0.07 ± 0.00 ^{a}	0.06 ± 0.01 ^{a}	0.08 ± 0.04 ^{a}	P=0.6924 ns
	Acutissimin B	0.04 ± 0.01 $^{a\ 1}$	0.06 ± 0.02 $^{a\ 1}$	0.13 ± 0.05 $^{ab\ 12}$	0.16 ± 0.06 $^{ab\ 12}$	0.24 ± 0.10 $^{a\ 2}$	0.12 ± 0.04 $^{ab\ 12}$	0.15 ± 0.12 $^{ab\ 12}$	P=0.1846 ns
	UEA	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.04 ± 0.05 ^{a}	0.06 ± 0.07 ^{a}	0.01 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	P=0.5541 ns
Sig. level		P=0.0023 **	P=0.0001 ***	P=0.0001 ***	P=0.0001 ***	P=0.0003 ***	P=0.0001 ***	P=0.0001 ***	

Data are presented as mg phenolic / g FW.

Per. - Pericarp; Int. - Integument; MeOH - Methanol; EtOH - Ethanol; UEA - Unidentified ellagic acid derivative.

a - Phenolics identified and quantified by HPLC at 270nm from calibration curves generated on the HPLC with pure standard.

For each one of the tissues considered, means with a common letter (within each column) and a common numeral (within each line) are not significantly different (P>0.05), according to Duncan New Multiple Range Test. ** and ***, significant at P < 0.01 and P < 0.001, respectively.

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CHAPTER 7

BACKGROUND: Chestnut fruits are highly regarded and widely consumed throughout Europe, America and Asia. Various commercial forms are available e.g. fresh and industrially processed. There have been various reviews on the composition of chestnut fruits but there has not been a comprehensive review of the different health benefits that this fruit can provide.

RESULTS: This review is focused on the composition and associated health effects of European fresh chestnut (*Castanea sativa* Mill.) fruits and their home-processed and industrial products e.g. boiled, roasted, frozen, and "marron glacées". We also expand the knowledge of chestnut uses by presenting data for other chestnut materials that have potential applications as new foods, as sources of antioxidants, and as sources of other useful bioactives.

CONCLUSION: There is considerable literature data on nutrients in fresh chestnut fruits but less information on bioactive non-nutrients such as phenolics. Chestnuts are mostly consumed as processed forms, and the different types of processing clearly affects the nutrient and non-nutrient composition of the fruits. The benefits that this fruit can provide for human and animal health are numerous, but it is clear that improvements can be made for both production and quality of chestnut products e.g. genetic selection and optimizing industrial processing.

Keywords: *Castanea sativa*; home cooking; industrial processing; proximate analyses; phytochemicals; health.

Composition of European Chestnut (*Castanea sativa* Mill.) and Association with Health Effects: Fresh and Processed Products

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7.1 Introduction

Chestnut fruits are an important food in many European countries. In 2008 the production of these nuts was 22 000t for Portugal, 55 000t for Italy, 6 258t for France, 15 000t for Spain, 55 800t for North and South America, 925 000t for China, 22 100t for Japan and 80 000t for Korea (FAOSTAT, 2010). The same source showed that from 2005 to 2008 the production area of chestnut in Europe increased from 81 511ha to 87 521ha, whereas the production area decreased in America (42 160ha to 40 190ha) and in Asia (252 991ha to 239 406ha). There have been various reviews and book chapters on composition of chestnut fruits but no overall review of the composition and health effects of fresh and processed European chestnut fruits, which is the main focus of this work. Thus the value of fresh chestnut fruits regarding their industrialization and how the processing affects composition and the economic value of co-products generated.

The compositional data presented in this review is from 102 cultivars (23 Portuguese, 69 Spanish, 5 Italian and 5 Greek) of *Castanea sativa* that have been previously studied. Since it would not be possible to show individual cultivar data we present ranges of the compounds found from all of these studies (Tables 7.2 to 7.6).

It is clear from several other studies that plant products are affected by different factors such as climatic conditions (CO_2, temperature, radiation and rainfall) and cultivation inputs (nutrient availability, minerals, and diseases and pests of the trees and of the fruits) and certainly the production of chestnuts is affected in a similar way. However, the focus of this review is on the raw materials composition, processing effects (home cooking and industrial) on the composition, and subsequent effects on health.

The European chestnut (*Castanea sativa* Mill.) probably had its origins in the Eastern Mediterranean region over 90 million years ago in the middle Cretaceous, later spreading throughout Europe during the Cenozoic period (Adua, 1999). Later between 900 - 700 BC deliberate chestnut tree cultivation occurred in the Asian regions of the Caspian and Black sea. After this time chestnut cultivation quickly spread to Greece and later the Romans discovered the benefits of this nut tree (Adua, 1999; Conedera *et al*., 2004). The genus *Castanea* belongs to the

angiosperm family Fagaceae which also includes other economically-important tree species e.g. *Quercus*.

Chestnut is geographically distributed in three major areas: Europe with *Castanea sativa* Mill., Asia with *Castanea creanata* Sieb. and Zucc. (Japan) and *Castanea mollissima* Bl. (China and Korea), and North America with *Castanea dentata* Borkh. (Bounous, 2005; Lang *et al*., 2006). Although the predominant cultivated species in Europe is *Castanea sativa* Mill., recently some hybrids resulting from the genetic crossing (rootstock) with other species of chestnut (*Castanea creanata* Sieb. and Zucc., and *Castanea mollissima* Blume) have been developed for improving nut production as well as increased resistance to the ink disease and the chestnut tree cancer (Ferreira-Cardoso, 2002).

7.2 Chestnut Uses and Co-Products

Fresh chestnut fruits are rarely consumed raw. They are processed in various ways, at home or at an industrial scale to improve the organoleptic properties (aroma, flavour, texture), the digestibility of the fruits i.e. making nutrients more bioavailable, and the shelf-life of various products from the industrial processes. After collection all cultivars are ready for consumption; however certain cultivars are more appreciated because of the organoleptic properties, although calibre and maturation time are also factors considered. Cultivars producing fruits with a polyspermy (the number of cotyledons/seeds inside the chestnut fruit) percentage over 12% are well accepted, but in general consumers have a noticeable preference for the “marron” chestnut fruits (monospermic). A calibre that does not exceed the 100 fruits/kg is preferred, because the bigger chestnuts are more appreciated (Desmaison and Adrian, 1986; Ferreira-Cardoso, 2002).

7.2.1 Home Cooking

Previous analysis presented data on the modification of structure and digestibility of chestnut starch upon cooking showing significant changes in the macro-molecular structure of this polysaccharide (Pizzoferrato *et al*., 1999). Bounous *et al*. (2000) studied the effect of different types of treatments (raw, dry, boiled, roasted and flour) on the nutritional composition of Italian chestnut fruits and the data obtained revealed that when boiled these nuts gain humidity but lose about 25% of energetic value, while by roasting the available sugars can increase by 25% and the energy levels increase significantly (200 kcal/100g). A recent study presented data on the processing effects of roasting and boiling on primary and secondary metabolite composition of Portuguese chestnuts showing an increase in protein content, insoluble and total

dietary fibre for the roasted fruits, while the boiled chestnuts revealed lower protein but higher fat contents (Gonçalves *et al*., 2010). However, the same authors stated that the raw fruits presented higher contents of malic acid compared with the cooked chestnuts.

7.2.2 Industrial Processing

Chestnuts are available in various industrial forms such as *i*) frozen at -40 ºC, *ii*) sterilized in aluminum bags (116 ºC for 30 to 35 min after vacuum sealing), *iii*) tinned (with a preservative liquid), *iv*) stored in flasks (with a preservative liquid), or *v*) dried fruits. These forms are mainly used in gastronomy, commonly with roasted meats. The fruits destined for this purpose are peeled kernels with no presence of cracks, with good calibre, optimal storage capacity, and a good sanitary aspect after storage (Bergougnoux, 1978; López *et al*., 2004; Gounga *et al*., 2008). Other products (purees, chestnut creams, flours, soups or yogurts) are generated from healthy but lower quality chestnut fruits; lower calibre, polyspermic and broken. This is an alternative and economically profitable way of increasing the valorisation of chestnut products and decreasing the levels of waste materials resulting from the industrial processing.

Chestnut fruits are sold fresh during the production season and in hard winter regions are often the only fresh product available. However, these fruits are now increasingly available as frozen products. Unfortunately there are only a few publications that describe the processing stages of industrial processing, for example De Vasconcelos *et al*. (2009a, 2009b and 2010). Frozen chestnuts are one of the major forms of this food and therefore these fruits are available all year round to the consumers. These studies showed that the industrial processing has positive and negative effects on the chestnut fruit nutrients. The positive effects on the fruits include the extension of the shelf life and increases from stage A (fresh) to stage D (frozen) in NDF (12.7-19.9 and 16.3-18.2g $100g^{-1}$ DM), total phenolics (6.6-12.0 and 10.6-16.9mg g^{-1} FW), Thr (4.9-6.8 and 3.6-7.9mg $100g^{-1}$ FW) and Ala (3.3-6.5 and 4.0-5.7mg $100g^{-1}$ FW) in 2006 and 2007, respectively, as well in δ-tocopherol (0.3-0.4µg g^{-1} FW in 2006) and γ-tocopherol (25.2-27.3µg g^{-1} FW in 2007). The negative effects were reductions in the levels of total starch (53.8-51.3 and 54.9-51.0g $100g^{-1}$ DM), Glu (14.7-9.2 and 13.9-10.9mg $100g^{-1}$ FW) and ascorbic acid (6.4-5.4 and 7.2-5.1mg $100g^{-1}$ FW) in 2006 e 2007, respectively, and also of fat (2.9-2.5g $100g^{-1}$ DM in 2007) (De Vasconcelos *et al*., 2009a, 2009b and 2010).

Through industrial processing, and specifically generation of frozen chestnut products, the losses related to dehydration are drastically decreased conferring to the fruits a long-term (out of season) availability and compositional stability. As part of industrial processing Hwang *et*

al. (2001) previously analysed the physicochemical factors related to the automatic pellicle removal in 14 Korean chestnut (*Castanea crenata*) varieties. These authors stated the existence of a negative correlation between the tannin content of the integument and the peeling ratio.

The kernels used for the production of sweets have to be, as previously described, in optimal condition after storage and have a good calibre. Some chestnut fruits are infiltrated with increasing concentration sugar solutions and conserved in flasks or metallic protected bags. Others can be added to bottles containing alcoholic beverages, allowing the sugar in the fruit to sweeten the liquor for a period of 6 to 12 months e.g. brandies, cognacs or digestive liquors. In France, Italy, Switzerland and Spain the most appreciated transformed chestnut fruits are the "marrons glacées". The chestnuts are submerged in a sugar-rich solution and then covered with glucose. The fruits are then cooked in an oven at 300 ºC for 1 to 2 minutes to crystallize the sugar (Bergougnoux, 1978; López *et al*., 2004). Other authors performed nutritional and microbiological evaluations of freeze-dried chocolate-coated (dark and milk) Chinese chestnut (*Castanea mollissima*) fruits showing high contents of sucrose, protein, minerals and also moisture contents suitable for industrial processing (Gounga *et al*., 2008). According to these authors this type of processing allows the decrease of microbiological loads and therefore extending the shelf lives of the chestnut products.

Studies have been performed evaluating the enzymatic hydrolysis of chestnut products (steamed fruit and purée) with mixtures of α-amylase and amyloglucosidase in order to develop the fruits as sources of glucose for different fermentation of distilled spirits and glucose syrups (López *et al*., 2004; López *et al*., 2005). Other authors evaluated the potential of the fruits to produce chestnut-based chips (Di Monaco *et al*., 2010) and the effect of drying conditions on chestnut flours and fruits (Moreira *et al*., 2005; Correia *et al*., 2009).

7.2.3 Other Chestnut Tissues Products

The Romans first discovered the large potential of chestnut trees, not only for fruit production but they also used the bark, leaves and flowers in pharmacopoeia. One of the founders of pharmacopoeia, Dioscoride Pedanio who worked in Nero's court, attributed to chestnut astringent, antitoxic and tonic characteristics (Adua, 1999).

Chestnut flower honey is much appreciated since early times by the Romans, positively described for its pleasant and mildly bitter taste (Adua, 1999). Chestnut honey is also used for dressing chronic wounds, burns or skin ulcers due to its antibacterial activity. Recent studies of chestnut honey reveal significant contents of compounds with antioxidant and antimicrobial

activities such as phenolics, flavonoids and quinoline alkaloids (Figure 1; Cordella *et al*., 2003; Küçük *et al*., 2007; Daher and Gülaçar, 2008; Beretta *et al*., 2009; Truchado *et al*., 2009).

Chestnut trees are tannin-rich with high levels of both procyanidins (condensed tannins) and ellagitannins (hydrolyzable tannins). This property has been exploited by using the wood for wine barrels and addition of wood chips directly to the wine; the leaching of these tannins from the wood provides the characteristic organoleptic properties in matured wines and brandies (Canas *et al*., 1999; Simón *et al*., 2009). The bark and wood of chestnut trees are the prime sources of tannins for leather processing; transformation of hides and skins into leather and as re-tanning agents (Korel and Balaban, 2009). During the tanning process the polyphenols are involved in the introduction of additional cross-links into collagen, which binds the active groups of the tanning agents to groups of the collagen of the skin (Cassano *et al*., 2003).

The chestnut shell resulting from the fruit peeling process is used in some countries as fuel (Spain) and is also a rich source of phenolics and antioxidants (Hwang *et al*., 2001; Calliste *et al*., 2005; Barreira *et al*., 2008; Vázquez *et al*., 2009; Živković *et al*., 2009). These antioxidants and other components present in shells, wood and leaves are used in animal feeds (for details see HEALTH EFFECTS - ANIMALS section) and could also be used as additives to human foods, allowing the recycling and decrease of vegetable waste together with a more environmentally friendly production of food products (Calliste *et al*., 2005; Barreira *et al*., 2008; Vázquez *et al*., 2009; Laufenberg *et al*., 2003).

7.3 Proximate Composition of Chestnut Fruits

7.3.1 Recommended Daily Intake (RDI) Values

The RDI values for macronutrients, minerals and vitamins are shown (Table 7.1) so that these values can be compared with the compositional data for chestnut fruits, to further demonstrate their nutritional benefits as part of a healthy balanced diet (Anonymous, 2004). In order to compare the RDI values with the composition of the specific components identified in chestnuts we have estimated the percent contribution from consuming 100g fresh weight of chestnut fruits as follows - using the dry matter data from Table 7.2 we calculated the mean of all the ranges (47.65g dry matter in 100g fresh chestnuts) and used this to convert the means of the ranges of the individual nutrients, energy, vitamins and minerals to a fresh weight basis and then used these values to compare with the RDI values (calculating the percentage contribution to the RDI).

7.3.2 Basic Composition

There are various simple (Bounous *et al*., 2000; Ertürk *et al*., 2006) and detailed studies (Tables 7.2 to 7.6) that have analyzed the chemical composition (proximate analyses) of chestnut fruits.

Chestnut fruits contain various nutrients (starch, free sugars, proteins, lipids/fat), vitamins, and minerals that are important for health (Tables 7.2 to 7.6). Chestnut fruits are mainly composed of carbohydrates, primarily starch (Table 7.2). The free sugar sucrose (saccharose) can be up to one third of the total sugars. Previous analyses revealed the presence of various mono- and di-saccharides (glucose, fructose, sucrose and maltose) (Table 7.2), that are very important for the assessment of the chestnut fruit commercial quality (De Vasconcelos *et al*., 2010; Bernárdez *et al*., 2004; Attanasio *et al*., 2004; Míguelez *et al*., 2004). Previous studies in Spanish and Italian chestnut cultivars have shown high contents of starch and additionally studies have been performed on Italian chestnuts to evaluate the specific contents of amylose and amylopectin (Pizzoferrato *et al*., 1999; Attanasio *et al*., 2004; Pereira-Lorenzo *et al*., 2006), which accounts for 33% and 67% of the starch content, respectively. Both of these forms of starch have positive health effects providing energy (catabolism of both amylose and amylopectin into glucose) and positive effects on gut functions due to bacterial catabolism of amylopectin-derived dextrins into short chain fatty acids (SCFA) (Pizzoferrato *et al*., 1999; Nichols *et al*., 2003).

The popularity of dietary ingredients used to improve human health has been increasing in the last few decades (Gibson, 2008). One of the most common, associated with positive health effects, is dietary fibre that consists of the remnants of edible plant cells polysaccharides, lignin and other substances resistant to digestion by the alimentary human enzymes. Dietary fibre is associated with stimulation of *Bifidobacteria* and *Lactobacillus* in the intestine, decreases in cholesterol levels, reduction of the risk of cardiovascular diseases, positive regulation of insulin response, increases in anticancer mechanisms, and positive effects on metabolism of blood lipids (Prosky, 2000). When dietary fibre is fermented by colonic bacteria short chain fatty acids (SCFA) are produced and these contribute to the maintenance of colonic integrity and metabolism. They are also potential therapeutic agents in health diseases such as various forms of colitis, antibiotic-associated diarrhoea and colon cancer (Cook and Sellin, 1998). From the data presented in Table 7.2 the mean of total carbohydrates/100g fresh chestnut fruits (starch, NDF, ADF and free sugars) was estimated to be 44.7g. This represents approximately 34% of

the RDI for females and males and therefore chestnuts are a good source of dietary carbohydrates and fibre.

Previous studies of Portuguese cultivars has revealed that chestnuts contain very little amounts of crude fat content which is low in saturated fatty acids and high in unsaturated fatty acids (Table 7.2; Borges *et al*., 2007). Chestnut fruit dietary contributions to total fat and PUFA are relatively low. Chestnut fat contains significant levels of mono-unsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), which are known for their anticancer effects and their association with a decreased risk of sudden death related to cardiovascular diseases and neurological function disorders (Hardaman, 2002; Whelan and Rust, 2006).

The energetic value (crude energy) of chestnuts, as in any other food source, comes from the oxidative degradation of carbohydrates, proteins and lipids. Not all of the released crude energy (CE) is available to the organism because a part of this energy leaves the body through the faeces, urine and as gases (mainly methane). The other part corresponds to the energy that is available to the metabolic mechanisms that allow the maintenance and thermoregulation of the organism. Few analysis of the crude energy in chestnut kernels were done, but they reveal significant values showing that the chestnuts can be used as an important source of dietary energy (Table 7.2; De Vasconcelos *et al*., 2009a; Barreira *et al*., 2009a). From the data presented in Table 7.2 the mean energy/100g fresh chestnut fruits was estimated to be 198kcal. This represents, depending on the BMI, approximately 9.0 to 9.8% of the RDI for females and 7.7 to 8.8% of the RDI for males.

7.3.3 Crude Protein and Free Amino Acids

The protein content of food is an important factor to achieve a healthy and nutritionally-balanced diet (Table 7.2). From the data presented in Table 7.2 the mean of crude protein/100g fresh chestnut fruits was estimated to be 3.5g. This represents approximately 9.2% of the RDI for females and 7.6% of the RDI for males. The amino acids, that constitute the proteins in the diet, are of high importance. Amino acids are biologically active and have multiple functions in the body; as sources of energy and as precursors of proteins and various important molecules. Amino acids are divided in essentials (the ones that the human body does not synthesize in enough quantities, so they are necessary from the diet) and non-essentials (the human body is able to synthesize them in adequate concentrations). Total amino acids (including free and protein-derived), from acid hydrolyses studies of chestnut fruits, have been analyzed for European (Portuguese and Italian; *Castanea sativa*), American (*Castanea dentata*) and Chinese

(*Castanea mollisima*) chestnuts; the common predominant protein-derived amino acids were aspartic acid and glutamic acid. Besides proteins, plant tissues also contain free amino acids (Meredith *et al*., 1988; Borges *et al*., 2008). There are some reports of measurements for free amino acids in *Castanea sativa* fruits (Table 7.3; Desmaison *et al*., 1984; De Vasconcelos *et al*., 2007). The predominant free amino acids in a recent study were aspartic acid, asparagine and glutamic acid (De Vasconcelos *et al*., 2009b).

According to Tixier and Desmaison (1980), chestnut fruits (*Castanea sativa*) also contain significant amounts (50 to 236 mg $100g^{-1}$ DM) of γ-amino butyric acid (GABA - derived from Arginine), which is an important neurotransmitter in the central nervous system. L-glutamic acid decarboxylase located in the cerebrum and hypothalamus, with pyridoxine (vitamin B_6) as its co-enzyme, converts L-Glu to GABA. One of the most common consequences of the absence of vitamin B_6, and the associated poor synthesis of GABA, is convulsions in children (Ferreira-Cardoso, 2007). GABA acts by decreasing the neuron activity calming the body and in combination with niacinamide and inositol prevents anxiety-related messages to reach the motor centers of the brain by filling the receptor site (Korel and Balaban, 2009).

7.3.4 Antioxidant and Other Vitamins

Vitamins are also essential in a balanced diet for various health related effects (Table 7.1). An essential compound for human health, that humans cannot synthesize, is vitamin C (ascorbic acid). The biochemical and molecular role of vitamin C (Figure 7.1) in the human body is mainly as an antioxidant, as part of a redox cycle with dehydroascorbic acid, and two well known consequences of vitamin C deficiency are the diseases scurvy and anaemia (Ching and Mohamed, 2001; FAO/WHO, 2004). Previous analysis also report that vitamin C functions as an important antioxidant in the mitochondria of human colon cells (Wenzel *et al*., 2004). The major lipid-soluble antioxidant in the cell is vitamin E (Figure 7.1), and is exclusively obtained from the diet. This vitamin has a direct antioxidant effect for polyunsaturated fatty acids, low density lipoprotein and other cell membranes components, protecting them from oxidation by free radicals and vitamin E has been associated with reducing the risk of cancer and cardiovascular diseases.

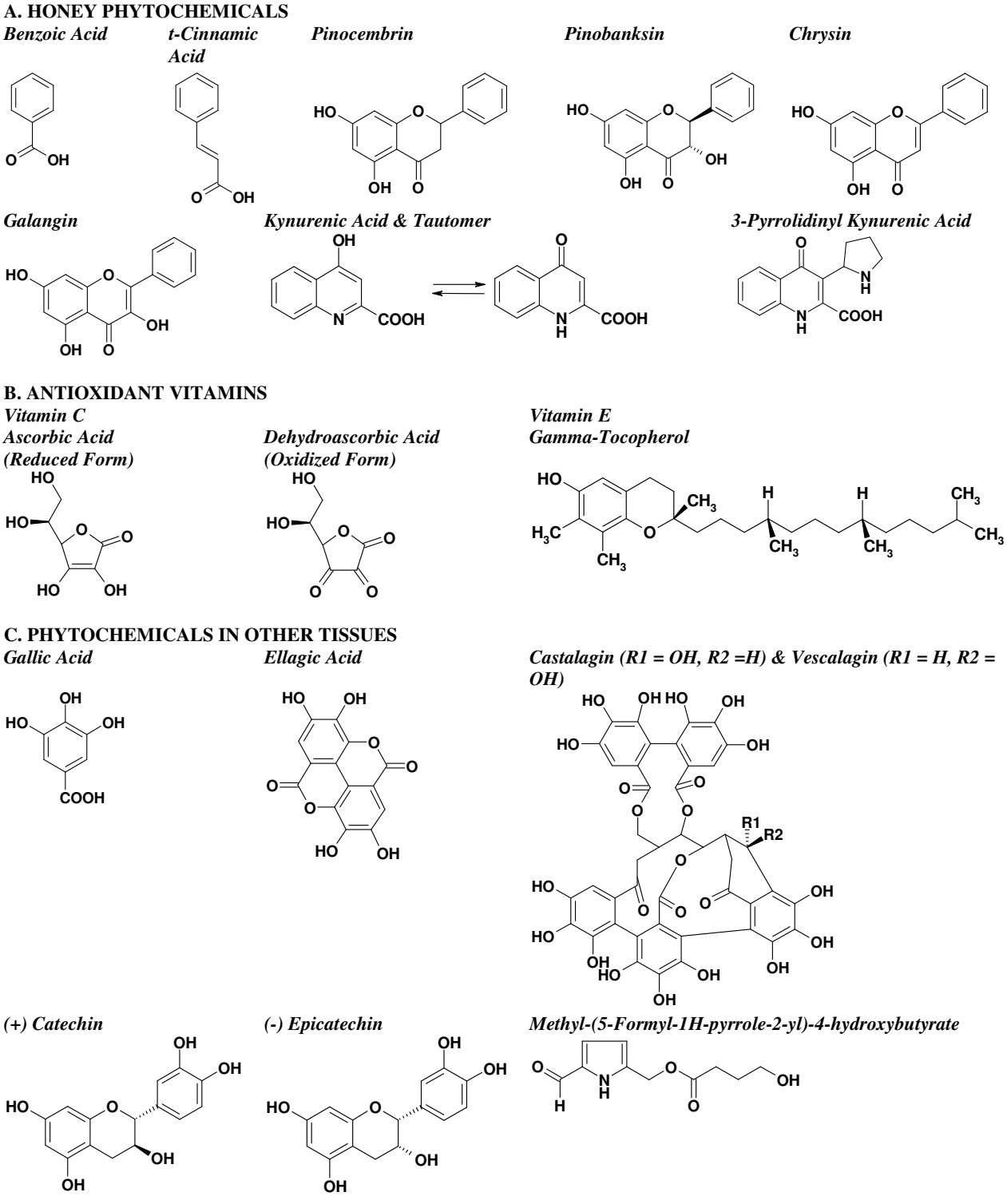

Figure 7.1 - Phytochemicals and vitamins from various tissues of *Castanea sativa* Mill.

The compositional analysis of these vitamins in chestnut fruits has been reported previously (Table 7.4; Peña-Mendez *et al*., 2008; Barreira *et al*., 2009b; De Vasconcelos *et al*., 2010). From the data presented in Table 7.4 the mean vitamin E / 100g fresh chestnut fruits was 1.9mg representing 12.7% of the RDI for both females and males. From the data presented in Table 7.4 the mean vitamin C / 100g fresh chestnut fruits was 15.6mg representing 20.8% of the RDI for females and 17.3% for males. This means that chestnuts are good dietary source of both vitamins E and C. Other vitamins previously reported in raw chestnut kernels are vitamin A (1µg $100g^{-1}$ FW: greater than RDI) which is important for the visual system, growth, epithelial cellular integrity, immune function and reproduction; thiamine (B_1 - 0.14mg $100g^{-1}$ FW: 12.7% of RDI for females and 11.7% for males) which is a coenzyme in carbohydrates and branched-chain amino acids metabolism; riboflavin (B_2 - 0.02mg $100g^{-1}$ FW: 1.8% of RDI for females and 1.5% for males) which is a coenzyme in redox reactions; niacin (B_3 - 1.10mg $100g^{-1}$ FW: 7.9% of RDI for females and 6.9% for males) is a precursor of important co-factors of enzymes and proteins in the electron transport chain i.e. NAD^+/NADH and $NADP^+$/NADPH; pantothenic acid (B_5 - 0.48mg $100g^{-1}$ FW: 9.6% of RDI for females and males) is a constituent of coenzyme A and involved in fatty acid metabolism); pyridoxine (B_6 - 0.35mg $100g^{-1}$ FW; 26.9% of RDI for females and males) which is a coenzyme in amino acids, glycogen and sphingolipid metabolism; folate (58µg $100g^{-1}$ FW: 14.5% of RDI for females and males) is an important co-factor of enzymes involved in various metabolic pathways, in the formation of red blood cells, in DNA synthesis and repair, and may also be important in various neurological functions) (FAO/WHO, 2004; Korel and Balaban, 2009).

7.3.5 Minerals

The content of minerals found in the chestnut fruits are not only associated with the genotype and weather conditions, but also related to the mineral composition of the soil where the chestnut trees were cultivated. The minerals contents were previously analyzed in chestnut fruits revealing important macro-elements (Ca, P, K, Mg and S) with potassium representing the majority of this group and also important micro-elements (Fe, Cu, Zn e Mn) (Table 7.5; Ferreira-Cardoso, 2002; De Vasconcelos *et al*., 2010; Pereira-Lorenzo *et al*., 2006; Borges *et al*., 2008). The increasing concern for a healthy nutrient-rich diet has become an important topic of public health and this includes a balanced mineral intake (Table 7.1). Calcium is associated with essential biological functions such as conferring rigidity to the skeleton. From the data in Table 7.5 the mean Ca content per 100g fresh chestnut fruits was 23mg, representing more than the

RDI for both females and males. Magnesium is an important cofactor of many enzymes, and is also involved in protein, RNA and DNA synthesis, and maintenance of the electrical potential of nervous tissues and cell membranes. From the data in Table 7.5 the mean Mg content per 100g fresh chestnut fruits was 35mg, representing 10.9% of the RDI for females and 8.3% for males. Micro-minerals also play important roles in health e.g. iron is important for the transport of oxygen through red blood cell haemoglobin and both iron and zinc are essential components of various enzymes that participate in the synthesis and catabolism of many nutrients and xenobiotics (Tontisirin and Graeme, 2004). From the data in Table 7.5 the mean Fe content per 100g fresh chestnut fruits was 3mg, representing 16.7% of the RDI for females and 37.5% for males. A recent study stated that daily zinc supplementation (capsules with multivitamins and 7mg of Zn) may positively affect the mood states in young women, showing a significant reduction in anger and depression with regular Zn intake (Sawada and Yokoi, 2010). From the data in Table 7.5 the mean Zn content per 100g fresh chestnut fruits was 0.9mg, representing 11.3% of the RDI for females and 8.2% for males. Chestnuts also contain other important minerals representing the following percentage contributions to the RDI values: P (89mg/100g FW - 12.7% of the RDI for females and males), K (465mg/100g FW - 9.9% of the RDI for females and males), Na (8mg/100g FW - 0.5% RDI for females and males), Cu (0.8 mg/100g FW - 88.9% of RDI for females and males), Mn (3.2mg/100g FW) and Se (0.3mg/100g FW) which are greater than the RDI for females and males.

7.4 Pigments and Secondary Metabolites

7.4.1 Pigments

A previous study analyzed the pigments (carotenoids and chlorophylls) contents in chestnut fruits presented in a USDA report and only beta-carotene (pro-vitamin A), lutein and zeaxanthin were detected; there were no chlorophylls or related derivatives present in the fruits (Table 7.6; USDA, 2008). However there are other studies that have analysed pigments in other chestnut tissues. These studies focused on the analyses of carotenoids and chlorophylls in leaves of Portuguese chestnut, showing high levels of these photosynthetic pigments, as well as other data revealing a significant carotenoid content in French chestnut (*Castanea sativa* Mill.) wood (Table 7.6; Masson *et al*., 1997; Gomes-Laranjo *et al*., 2006). The carotenoids content in fruit and leaves could also provide benefits for human health through different mechanisms, such as

direct antioxidant effects, redox sensitive cell signalling and vitamin A signalling pathways (Elliot, 2005).

7.4.2 Total phenolics, Specific Phenolics and Tannins

Various aromatic compounds including simple phenolics and more complex tannins have been detected in chestnut tissues (see Figure 7.1 for examples). The chestnut fruits content of phenolics (gallic and ellagic acid) has been previously analyzed (Table 7.6; De Vasconcelos *et al*., 2007; De Vasconcelos *et al*., 2009b). These acids have been linked with various positive health effects such as antioxidant effects, decreases in the risk of cardiovascular diseases, anticancer mechanisms and anti-inflammatory properties (Veluri *et al*., 2006; Mertens-Talcott *et al*., 2006; Hooper *et al*., 2008). Many *Castanea* tissues are rich in both simple phenolics and more complex tannins. Low levels (0.01-0.02 mg $100g^{-1}$ edible portion) of monomeric, dimeric and trimeric procyanidins (condensed tannins) have been reported in chestnut fruits (Pascual-Teresa *et al*., 2000; Gu *et al*., 2004). However other chestnut tissues, e.g. leaves, wood and bark, have much higher levels of these phenolics (Table 7.6; Barreira *et al*., 2008; Vázquez *et al*., 2008).

7.4.3 Alkaloids

Plants have a high capacity to synthesize a large variety of low molecular weight compounds, the secondary metabolites (phytochemicals). The function of these compounds is still not evident but in the past few decades experimental and circumstantial evidence show that they are vital in plant defence mechanisms (Wink, 1999).

Alkaloids are an important class of secondary metabolites that are biosynthesized in many plants but their biosynthesis, regulation and specific roles are not fully understood. The majority of alkaloids are derived from tyrosine, phenylalanine, anthranilic acid, tryptophan/tryptamine, ornithine/arginine, lysine, histidine and nicotinic acid. However, they can also be derived from purines, terpenoids and steroids (Roberts and Strack, 1999). A new pyrrole alkaloid, methyl-(5-formyl-1*H*-pyrrole-2-yl)-4-hydroxybutyrate, has been identified in *Castanea sativa* seeds (1mg $100g^{-1}$ DM) (Figure 7.1; Hiermann *et al*., 2002). Pyrrole alkaloids have also been found in other plants and marine sponges (Pinder, 1989; O'Hagan, 1997 and 2000; Walsh *et al*., 2006). Alkaloids, of various classes, are known to have important medicinal effects in humans (Wink, 1998; Schmeller and Wink, 1998; Aniszewski 2007a and 2007b). Many

important drugs are alkaloids or are derived from alkaloids. There have been no reports on the positive or negative biological effects of the chestnut alkaloid in animals or humans.

7.4.4 Flavour Compounds

Flavour is an important organoleptic property of foods and cooking usually leads to positive changes in flavour; this is the case for chestnut fruits. However, the type of cooking determines whether potential toxic compounds are formed e.g. the formation of acrylamide during high temperature cooking. Maillard reactions occur between amino acids and reducing sugars during heating or cooking processes, leading to the development of colour (non-enzymatic browning) and volatile heterocyclic compounds that are involved in flavour (Morini and Maga, 1995). This process may also be responsible for the modification of polysaccharides and proteins in chestnut fruits after cooking. Previously identified volatile compounds in roasted chestnuts included gamma-butyrolactone (faint, sweet, caramel flavour) and furfural (sweet, woody, almond, fragrant, baked bread flavours) (Krist *et al*., 2004). Other work identified considerable amounts of acrylamide in roasted chestnuts that might have negative effects in health, being classified as a probable human carcinogen and also known as a neurotoxin (Karasek *et al*., 2009).

7.5 Health Effects - Animals

A previous study on chestnut fruits, in relation to the fattening of Duroc pigs (non-castrated males), revealed that chestnuts are a good food supplement contributing to the energy requirements of the pigs and improving the sensorial taste of the meat and ham. However, the percentage of incorporation of chestnut fruits into the pig´s diet cannot be more than 30% w/w of the DM otherwise negative effects on the pig performance (growth and fattening), carcass composition and meat quality are observed. The feeding of chestnuts also reduces the nitrogen composition in urine and faeces from the pigs and therefore has an additional positive environmental effect - reducing the generation of deleterious nitrogenous substances from pig production (Ferreira-Cardoso, 2002). Chestnut tannin extracts are being evaluated as potential replacements for antibiotics in animal production. Previous studies showed that the addition of hydrolysable tannins extracted from *Castanea sativa* wood to the diets of sheep and goats (0.11%), chickens (0.20%) and rabbits (0.45%) may improve their health and even increase their life spans. These tannins may act by preventing or eliminating the gut colonization of parasites (Zimmer and Cordesse, 1996; Schavione *et al*., 2008; Zoccarato *et al*., 2008).

7.6 Health Effects - Humans

7.6.1 Chestnut and Allergies

Chestnut pollen can cause type 1 (contact allergy, immediate hypersensitivity reaction) allergies in some humans (pollinosis) (Hirschwehr *et al*., 1993; Teuber *et al*., 2003; Hoffmann-Sommergruber and Mills, 2009). A previous study presented the molecular and immunologic characterization of the major allergen from the European chestnut - Cas s1 (Kos *et al*., 1993). However it is generally considered as having a low to moderate allergy risk, but is related to cross-reactivity with natural rubber latex allergens. Recent studies with chestnut fruits have identified the major allergens and in Korean test subjects the sensitization rate, from ingestion, was 3.2% (Lee *et al*., 2005). In the same study the IgE reactive proteins in chestnut were greatly reduced by simulated gastric digestion. Reduction of IgE reactive proteins was even more effective when digestion was performed with boiled chestnuts; probably due to protein denaturation during cooking and further denaturation in the stomach.

7.6.2 Beneficial Effects of Chestnut

Celiac (a.k.a. coeliac) disease is a debilitating polygenic disorder caused by intolerance (auto-immune reactions) to peptides derived from digestion of the cereal protein gluten (Green and Jabri, 2006). The symptoms of celiac disease include diarrhoea, irritable bowel syndrome, malabsorption syndrome leading to deficiencies in vitamins and minerals (Folate, vitamins E, D and K, and the mineral Fe), unexplained weight loss, and also auto-immune diseases (type 1 diabetes, billiary cirrhosis and Sjogren´s syndrome). It has also been associated with Down´s and Turner´s syndromes and various neurological disorders (Green and Jabri, 2006). Celiac sufferers have to use gluten-free foods, which limits the type of foods they can consume. Chestnut fruits do not contain gluten and thus there are many new products derived from chestnuts and chestnut flour that have been created to replace the wheat/cereal-containing foods. In addition, from the various proximate analyses, it is clear that chestnut fruits are a good source of essential dietary nutrients and minerals. The low crude fat content, in combination with the high poly-unsaturated fatty acids in this fat, makes chestnuts a very healthy food. The free sugars and high starch content also make chestnuts an energetically valuable food crop.

7.6.3 Chestnut as a Functional Food

From the various composition and health studies it is clear that chestnut fruits, and potentially other extracts from chestnut trees, have considerable potential as functional foods or as food ingredients e.g. chestnut polyphenolic extracts as a natural source of antioxidants and other beneficial compounds such as gallic and ellagic acids and the ellagitannins. It has previously been shown that many ellagitannins, including castalagin and vescalagin, have potent antitumor, antioxidant, antimicrobial and anti-malarial properties (Kasiwada *et al*., 1992; Cerdá *et al*., 2004; Seeram *et al*., 2005; Reddy *et al*., 2007).

7.7 General Comments - The Future for Chestnut

To increase the market value and consumption of chestnut fruits based on the potential positive health effects measured from compositional and human health studies, it is important to further promote the benefits of these fruits through consumer awareness campaigns and food fairs.

In terms of future research it is clear that there are several specific areas for improving both the production and quality of chestnut-derived materials. One of the major factors affecting the quality of chestnut products is climate stress e.g. increasing CO_2 and UV, decreasing water availability, increasing extreme weather events leading to increased pathogen and pest damage. By conserving and developing the genetic resources for *Castanea sativa* Mill. and related species (germplasm banks and wild species) and developing more effective selected breeding programs it should be possible to improve not only the resistance to the various stresses but also to improve nutritional and other economically-important traits. Another aspect related to chestnut quality, and specifically for the fruits, is the industrial processing methods. It is clear from recent studies that some aspects of processing decrease the nutritional value of the fruits. It is therefore necessary to identify which stages of processing are causing the biggest deleterious effects and develop more effective processing methods.

Finally, to further support health claims regarding chestnuts it will be necessary to perform human cell model studies (for basic evaluations on metabolism and gene expression e.g. associated with the induction of health-related genes and apoptosis of cancer cells) and also human intervention studies to evaluate the pharmacokinetics and health biomarkers associated with fruit consumption.

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Table 7.1 - Reference Daily Intake (RDI) values for macronutrients, vitamins and minerals (Anonymous, 2004).

	RDI	
	Females (19 to 50 years)	**Males (19 to 50 years)**
Carbohydrates (g/d)	130	130
Total Fibre (g/d)	25	38
Fat (g/d)	20-35*	
Fatty acids		
Linoleic	12	17
α-Linolenic	1.1	1.6
Energy (kcal/day)	2016 - 2202**	2254 - 2566**
Protein (g/d)	38	46
Vitamins (mg/d)		
A	0.7	0.9
C	75	90
E	15	15
Thiamine (B1)	1.1	1.2
Riboflavin (B2)	1.1	1.3
Niacin (B3)	14	16
Pantothenic acid (B5)	5	5
Pyridoxine (B6)	1.3	1.3
Folate	0.4	0.4
Minerals (mg/d)		
Ca	1.0	1.0
Cu	0.9	0.9
Fe	18	8
Mg	320	420
Mn	1.8	2.3
P	700	700
Se	0.055	0.055
Zn	8	11
K	4700	4700
Na	1500	1500

* Percent of energy; ** Low active individuals with 1.65m of height and body mass index (BMI) of 18.5 kg/m^2 and 24.99 kg/m^2.

Table 7.2 - Proximate analyses previously reported for chestnut fruits.

Dry Matter (g 100g^{-1} FW)	**Total Ash** (g 100g^{-1} DM)	**Crude Fat** (g 100g^{-1} DM)	**Crude Protein** [(1)] (g 100g^{-1} DM)	**Crude Energy** (Kcal 100g^{-1} DM)	**References**
40.1 - 50.9	-	1.0 - 1.7	5.6 - 10.9	-	Ferreira-Cardoso *et al*., 1993
35.6 - 47.3	2.2 - 2.9	0.9 - 1.7	5.4 - 9.5	-	Ferreira-Cardoso, 2002
49.3 - 46.4	2.3 - 2.5	-	-	-	Sacchetti and Pinnavaia, 2005
39.9 - 59.7	1.8 - 3.2	1.7 - 4.0	4.5 - 9.6	-	Pereira-Lorenzo *et al*., 2006
48.7 - 54.6	1.8 - 2.3	1.6 - 2.6	3.9 - 5.2	-	De Vasconcelos *et al*., 2007
46.7 - 53.6	1.5 - 2.2	1.7 - 3.1	4.9 - 7.4	-	Borges *et al*., 2008
-	-	1.9 - 4.4	-	410 - 428	De Vasconcelos *et al*., 2009a
45.4 - 48.1	1.5 - 1.9	1.6 - 1.8	5.0 - 6.5	401 - 402	Barreira *et al*., 2009a

Total Starch (g 100g^{-1} DM)	**Fibre** (g 100g^{-1} DM)				
	NDF	**ADF**	**ADL**	**Cellulose**	**References**
49.7 - 58.7	23.2 - 28.9	2.9 - 3.6	0.4 - 1.2	2.0 - 2.9	Ferreira-Cardoso *et al*., 1993
54.9 - 64.9	20.2 - 27.2	2.8 - 3.4	0.6 - 1.3	2.0 - 2.4	Ferreira-Cardoso, 2002
55.3 - 67.2	-	-	-	-	Sacchetti and Pinnavaia, 2005
42.2 - 66.5	9.4 - 28.5	2.3 - 4.5	-	-	Pereira-Lorenzo *et al*., 2006
57.5 - 64.9	12.7 - 17.5	2.5 - 3.5	0.2 - 0.4	2.3 - 3.1	De Vasconcelos *et al*., 2007
38.6 - 47.9	13.8 - 24.4	1.9 - 3.2	-	-	Borges *et al*., 2008
-	12.7 - 19.9	2.8 - 4.1	0.4 - 0.6	2.4 - 3.6	De Vasconcelos *et al*., 2009a
-	2.7 - 3.8	0.5 - 0.6	0.02	0.5 - 0.6	Barreira *et al*., 2009a

Free Sugars (g 100g^{-1} DM)				
Sucrose	**Glucose**	**Fructose**	**Maltose**	**References**
22.3 - 29.7	1.1 - 2.3	1.0 - 2.3	0.6 - 1.8	Attanasio *et al*., 2004
6.6 - 19.5	0 - 0.3	0.04 - 0.3	0	Bernárdez *et al*., 2004
8.8 - 14.9	0.04 - 0.6	0.1 - 0.8	0.1 - 1.4	De Vasconcelos *et al*., 2010

Fatty Acids (g 100g^{-1} Total Lipids)					
SFA	**C18:1**	**MUFA**	**C18:2**	**PUFA**	**References**
22.8 - 27.7	17.4 - 31.5	17.9 - 31.9	39.1 - 48.3	43.0 - 55.9	Ferreira-Cardoso, 2007
14.1 - 18.6	20.7 - 37.6	22.5 - 39.3	37.6 - 50.9	42.0 - 60.1	Borges *et al*., 2007
16.2 - 19.4	29.6 - 37.4	30.9 - 38.7	37.9 - 45.5	42.0 - 51.9	Barreira *et al*., 2009a
19.9	35.9	37.3	38.2	42.8	Korel and Balaban, 2009

[(1)] N Total x 5.3.

FW- Fresh Weight, DM- Dry Matter; NDF- Neutral Detergent Fibre, ADF- Acid Detergent Fibre, ADL- Acid Detergent Lignin; SFA- Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA- Polyunsaturated fatty acids.

Table 7.3 - Contents of free amino acids previously reported in chestnut fruits.

Free Amino Acids (mg 100g^{-1} FW)	**References**	
	De Vasconcelos *et al*., 2007	De Vasconcelos *et al*., 2009b
Essential		
Arg	4.5 - 48.2	0.5 - 1.7
Ile	4.4 - 14.8	0.3 - 1.4
Leu	0.8 - 5.8	0.3 - 1.0
Phe	1.3 - 9.6	0.5 - 1.8
Thr	-	3.6 - 10.0
Trp	-	0.3 - 2.2
Val	4.9 - 11.5	0.8 - 2.7
Non-Essential		
Ala	10.1 - 45.6	3.3 - 10.0
Asn	62.0 - 149.5	14.9 -36.4
Asp	22.1 - 76.4	2.3 - 11.3
Gln	6.9 - 16.5	2.2 - 7.3
Glu	27.4 - 109.8	4.6 - 14.7
Gly	-	-
Ser	2.7 - 7.1	1.4 - 3.8
Tyr	0 - 1.4	0.4 - 1.1

Table 7.4 - Contents of the antioxidant vitamins E and C previously reported for chestnut fruits.

Vitamin E (µg g^{-1} FW)					**Vitamin C (mg 100g^{-1} FW)**		**References**
Tocopherols			**Tocotrienols**				
α	**γ**	**δ**	**γ**	**δ**	**ASc[a]**	**DHASc[a]**	
-	-	-	-	-	30.8 - 36.3 [b]		Peña-Mendez *et al*., 2008
-	-	-	-	-	40.2 [b]		Korel and Balaban, 2009
0.02 - 0.1	3.8 - 4.8	0.2 - 0.3	1.4 - 3.9	0.01 - 0.04	-	-	Barreira *et al*., 2009b
-	4.1 - 27.3	0.2 - 1.0	-	-	4.2 - 7.2	3.1 - 6.8	De Vasconcelos *et al*., 2010
-	-	-	-	-	0.77 - 1.64 [bc]		Ribeiro *et al*., 2007

[a] ASc = Ascorbic Acid (Reduced Vitamin C), DHASc = Dehydroascorbic Acid (Oxidized Vitamin C).

[b] Content of total vitamin C (ascorbic acid + dehydroascorbic acid).

[c] Data are presented in DM basis as a range of the values from analyses of raw, roasted, boiled and fried samples.

Table 7.5 - Contents of minerals previously reported for chestnut fruits.

Minerals (mg 100g^{-1} DM)												References
Ca	**P**	**K**	**Mg**	**S**	**Na**	**Fe**	**Cu**	**Zn**	**Mn**	**B**	**Se**	
26.5 - 44.0	119.2 - 181.4	631.2 - 844.1	47.4 - 75.6	50.7 - 91.9	24.8 - 30.9	1.9 - 9.0	0.4 - 0.7	0.8 - 1.2	1.8 - 5.5	-	-	Ferreira-Cardoso *et al*., 1993
28.1 - 46.8	129.9 - 238.4	1016.5 - 1475.7	55.4 - 81.3	38.1 - 132.8	2.28 - 3.08	2.5 - 5.3	0.5 - 0.8	0.9 - 1.5	2.2 - 7.8	-	-	Ferreira-Cardoso, 2002
37.1	181.6	1202.7	67.2	80.2	2.7	3.3	0.7	1.1	4.7	-	-	Ferreira-Cardoso *et al*., 2005
26 - 72	68 - 305	789 - 1130	49 - 100	-	3.0 - 26.0	1.4 - 2.4	0.6 - 1.0	1.0 - 1.9	1.7 - 12.5	-	-	Pereira-Lorenzo *et al*., 2006
40.8 - 50.6	104 - 148	473 - 974	63.3 - 93.3	-	0.8 - 3.9	5.3 - 10.9	1.3 - 2.7	1.4 - 3.1	3.1 - 8.0	-	-	Borges *et al*., 2008
30.8 - 45.8	99.8 - 156.4	629.2 - 811.1	49.4 - 58.7	26.4 - 50.3	19.9 - 28.4	3.0 - 7.1	0.6 - 0.9	0.6 - 1.3	1.5 - 5.1	3.0 - 3.1	0.4 - 0.8	De Vasconcelos *et al*., 2010

Table 7.6 - Contents of phenolics and pigments previously reported for fruits and other chestnut tissues.

Tissue	Phenolics (mg g^{-1} FW)			Pigments (µg g^{-1} FW)						References
	Total Phenolics	**Gallic Acid**	**Ellagic Acid**	**Lutein**	**β-Carotene**	**Total Minor Carotenoids**	**Lutein Esters**	**Total Carotenoids**	**Total Chlorophylls**	
Wood	-	-	-	0.2	0.7	-	-	-	-	Masson *et al*., 1997
Wood	-	-	89	-	-	-	-	-	-	Bianco *et al*., 1998
Bark	-	-	0.7- 21.6 2.8 - 18.4 [a]	-	-	-	-	-	-	Vekiari *et al*., 2008
Leaves	-	-	-	-	-	-	-	14.7 - 22.6 [b]	78.2 - 119.6 [b]	Gomes-Laranjo *et al*., 2006
Leaves	21.5	-	-.	-	-	-	-	-	-	Barreira *et al*., 2008
Flowers	48.6	-	-	-	-	-	-	-	-	Barreira *et al*., 2008
Whole Shell	26.6 - 52.3	-	-	-	-	-	-	-	-	Vázquez *et al*., 2008
Outer Shell	25.4	-	-	-	-	-	-	-	-	Barreira *et al*., 2008
Outer Shell	-	-	0.04 - 0.2 0.7 - 6.0 [a]	-	-	-	-	-	-	Vekiari *et al*., 2008
Inner Shell	102.6	-	-	-	-	-	-	-	-	Barreira *et al*., 2008
Inner Shell	-	-	0.03 - 0.1 0.5 - 0.8 [a]	-	-	-	-	-	-	Vekiari *et al*., 2008
Fruit	21.1 - 36.7	2.8 - 9.1	2.7 - 9.6	-	-	-	-	-	-	De Vasconcelos *et al*., 2007
Fruit	-	-	-	0.1 [c]	0.1	-	-	-	-	USDA, 2008
Fruit	0.7	-	-.	-	-	-	-	-	-	Barreira *et al*., 2008
Fruit	-	-	0.05 0.05 [a]	-	-	-	-	-	-	Vekiari *et al*., 2008
Fruit	6.6 - 18.3	6.1 - 25.6	3.8 - 47.8	-	-	-	-	-	-	De Vasconcelos *et al*., 2009b
Fruit	-	-	-	0.1 - 0.6	0.01 - 0.1	0.02 - 0.2	0.1 - 0.4	0.23 - 1.30	-	De Vasconcelos *et al*., 2010

[a] The first values shown are for non-hydrolyzed contents, the second for acid hydrolyzed; [b] Values are presented as mg cm^{-2}; [c] lutein plus zeaxanthin.

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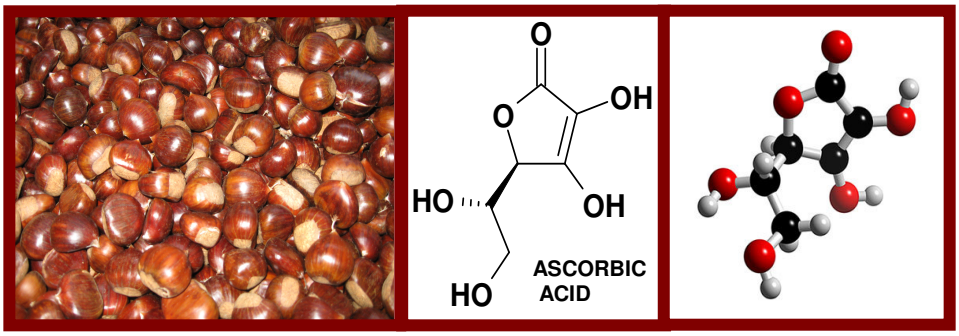

CHAPTER 8

CONCLUDING REMARKS

8.1 General Conclusions and Discussion

Chestnut fruits are a good source of several nutrients that are essential in a balanced diet. The previous compositional analyses reveal a high content of carbohydrates and significant amounts of vitamins, amino acids, proteins, dietary fibre, phenolics, pigments, minerals and although low in fat, these nuts reveal also low levels of saturated fatty acids and high of unsaturated fatty acids, known for their positive health effects. These facts justify the intensive compositional research performed in order to evaluate and improve the industrial processing of this nut. Moreover, we expect to give a contribution in order to support the increase of the cultivation of one of the major crops in the North-Eastern regions of Portugal, which represents an important income for the farmers of these areas particularly in the peasant farming systems. Chestnuts also represent a good and fresh source of nutrients during the hard winter season when fruit and vegetable products, other than *Brassica* species, are scarce.

It is clear from the new analyses presented in this thesis that industrial processing of chestnut fruits lead to both "positive" and "negative" changes in relation to biologically active components, both nutrients and health-promoting non-nutrients. Overall the industrial processing of chestnut fruits lead to increases in crude energy, fibre, phenolics, Thr, Ala and tocopherols and reductions in the levels of total starch, fat, Glu and vitamin C.

From the evolution of the processing, it was found that there were significant changes in the amounts of the analysed compounds, especially from stage A to B (post-storage) and from stage B to C (post flame peeling). The decrease in the starch contents, of the cultivars analysed in the three harvest years (2005, 2006, 2007) from processing stages A to B, may be explained by enzymatic catabolism by α-amylases (α-1,4-Endoglucosidases), first into maltose, then catabolism by various glucosidases to glucose. The glucose released can then be enzymatically-coupled with fructose forming more sucrose. This theory is supported by the visible increase of sucrose contents, from stage A to B, in the 2006 and 2007 harvests. Chestnut fruit starch consists of two forms of glucose polymers: amylose (33%) that is a linear polymer with few branches and amylopectin (67%) a complex 3D polymer with multiple branching (Attanasio *et al*., 2004; Nichols *et al*., 2003; Figure 8.1). Chestnut fruits also contain non-starch carbohydrates that are: *i*) mono- and disaccharides and soluble fibre; *ii*) oligo- and polysaccharides and insoluble fibre (dietary fibre). These compounds have extremely important positive health effects related to the production of short chain fatty acids (SCFA) through bacterial colonic fermentation of resistant starches that result inevitably from the digestion of amylopectin into limit dextrins (Cook and Sellin, 1998; Bird *et al*., 2000; Ao *et al*., 2007; Figure 8.1).

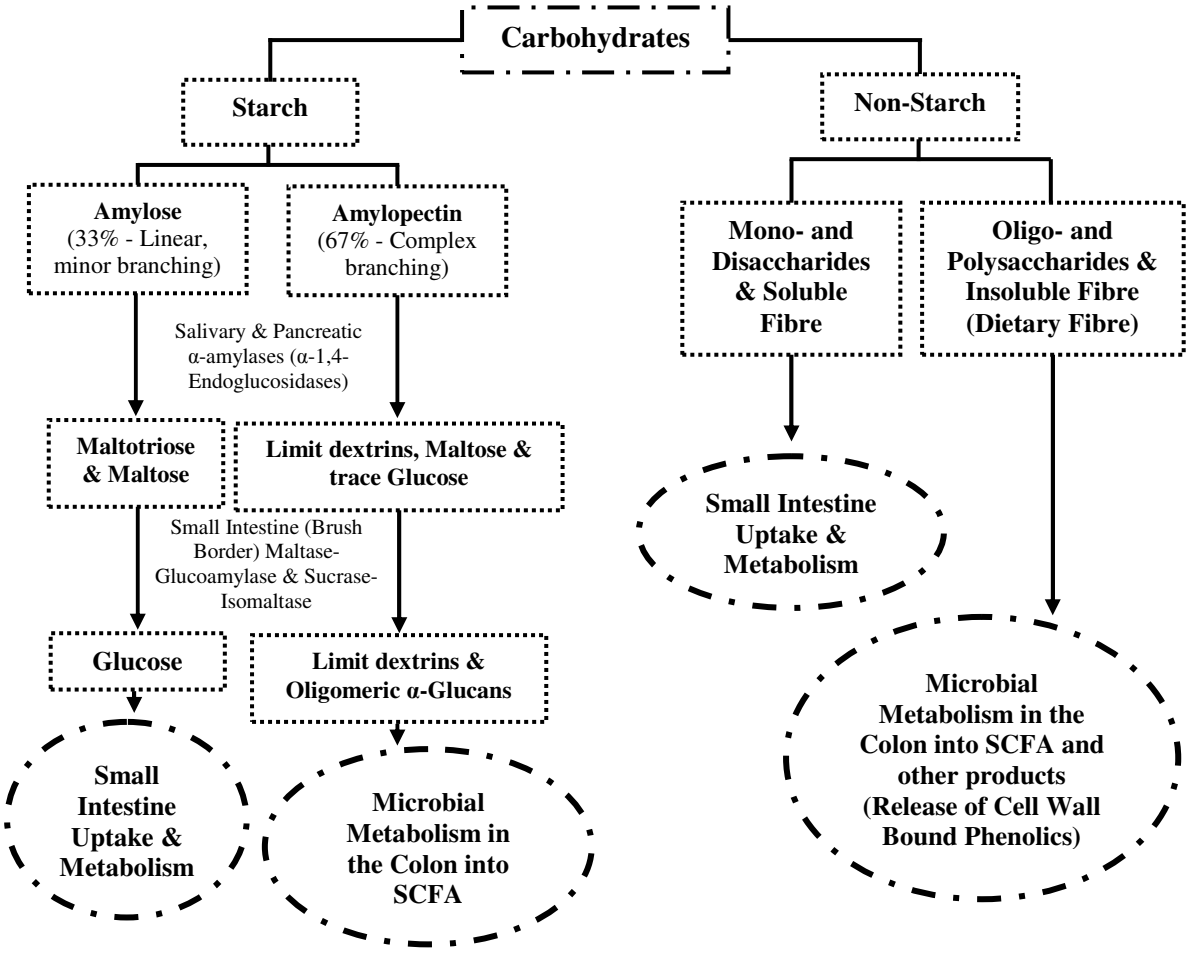

Figure 8.1 - Carbohydrate metabolic processes in the human body.

In addition another significant change was observed from stage A to B in the three harvests analyzed - a visible increase in total phenolics and ellagic acid contents, which may be due to ellagitannin decomposition. For example, catabolism of one molecule of vescalagin produces one molecule of vescalin and one molecule of ellagic acid, leading simultaneously to an increase in total phenolics (Folin Ciocalteau values) and specifically in ellagic acid contents (Niemetz and Gross, 2005; Gross, 2008). The increase revealed in the gallic acid values between these stages, in 2006 and 2007, may be related to *de novo* biosynthesis through the shikimic acid pathway (Figure 8.2) and/or catabolism of galloyl esters (precursors of ellagitannin biosynthesis) (Werner *et al*., 2004; Gross, 2008). In humans both ellagitannins and ellagic acid are further catabolised into urolithins and related aromatic metabolites that have been shown to have various positive health effects (Bialonska *et al*., 2009).

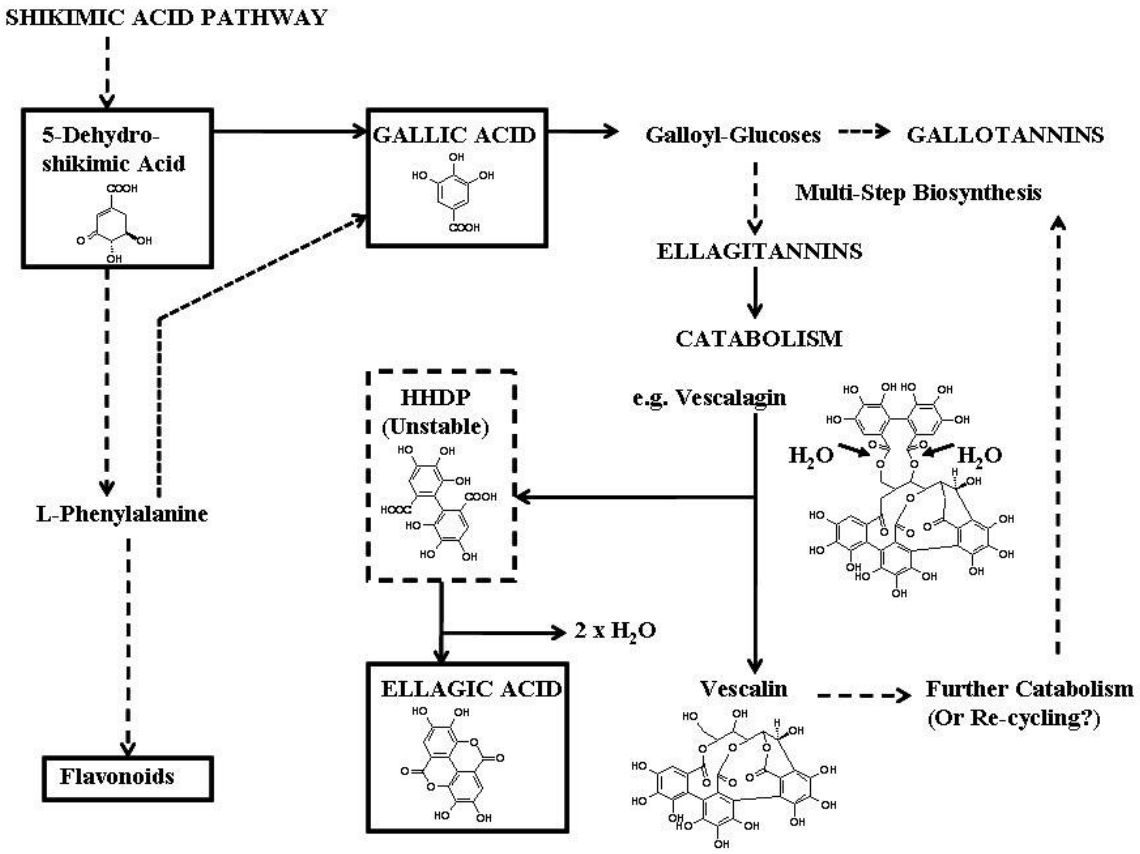

Figure 8.2 - Shikimic acid pathway and biosynthesis of hydrolyzable tannins.
Condensed tannins (including procyanidins) are synthesized via flavonoids, specifically flavan-3,4-diols and/or flavan-3-ols.

The losses observed in some constituents, between processing stages B and C, could be occurring via non-enzymatic thermal decomposition because some compounds are not stable to high temperature; they are thermo-labile. This was observed in the contents of vitamins E and C, carotenoids and some free amino acids (significant reductions in aspartic acid and glutamic acid). In chestnut fruits the form of vitamin E that is predominant is γ-tocopherol (Figure 8.3) and it significantly decreased from processing stage B to C of the 2006 and 2007 harvests that might be due to the above referred non-enzymatic thermal decomposition. Gamma(γ)-tocopherol has been shown to be important in the prevention of colon cancer and is superior to α-tocopherol in its health effects (Campbell *et al.*, 2003). In addition a combination of γ-tocopherol with α-tocopherol was far more effective than treatment with α-tocopherol alone as an anti-inflammatory agent (Reiter *et al.*, 2007). However, some researchers still consider α-tocopherol to be more important, but this depends on how the biological activity is expressed and also most of this data has come from rat studies (Clarke *et al.*, 2008).

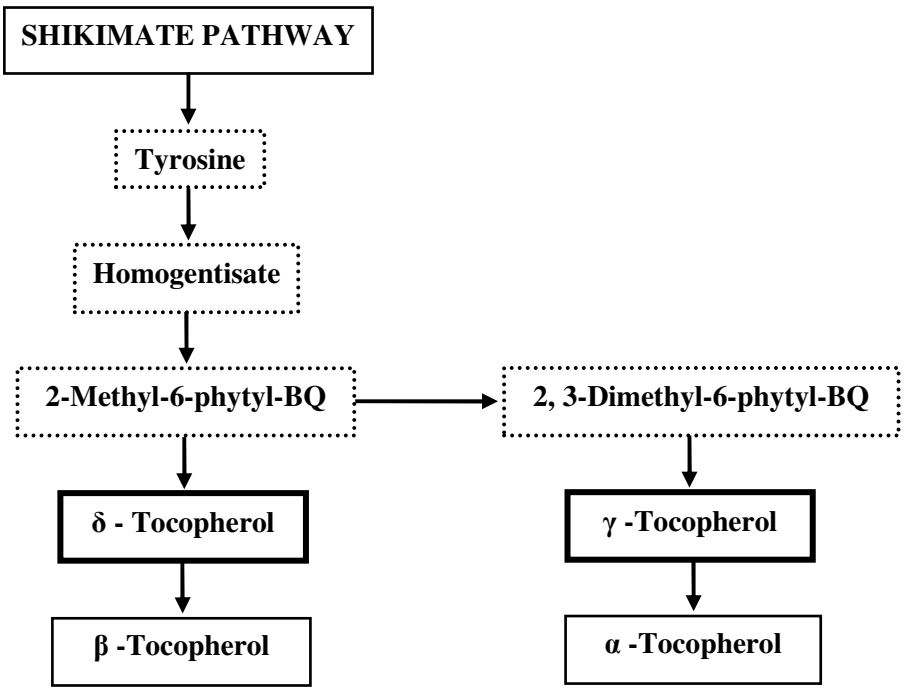

Figure 8.3 - Vitamin E biosynthetic pathway.

The vitamin C contents were quantified as ascorbic (Asc) and dehydroascorbic (DHAsc) acids. In the redox cycle Asc is first oxidized into mono-dehydroascorbic acid (MDHAsc), which can either be reduced back into Asc or oxidized further into DHAsc. DHAsc can then be irreversibly/non-enzymatically hydrolyzed into 2,3-Diketogulonic acid, which could explain the decreases in the total amounts of vitamin C (Asc and DHAsc) in chestnut fruits during processing (Chen *et al*., 2003; Valpuesta and Botella, 2004; Hancock and Viola, 2005; Wolucka and Van Montagu, 2007; Figure 8.4).

With respect to the losses of free sugars and free amino acids, Maillard reactions do not appear to be significant from stage B to C (reactions between reducing sugars and amino groups on free amino acids/proteins), since the levels of free sugars, including reducing sugars, increase. The contents of glucose and fructose revealed an increase from processing stage B to C that is consistent with catabolism/decomposition of the disaccharide sucrose into the monosaccharides glucose and fructose. This was especially visible in the 2007 harvest.

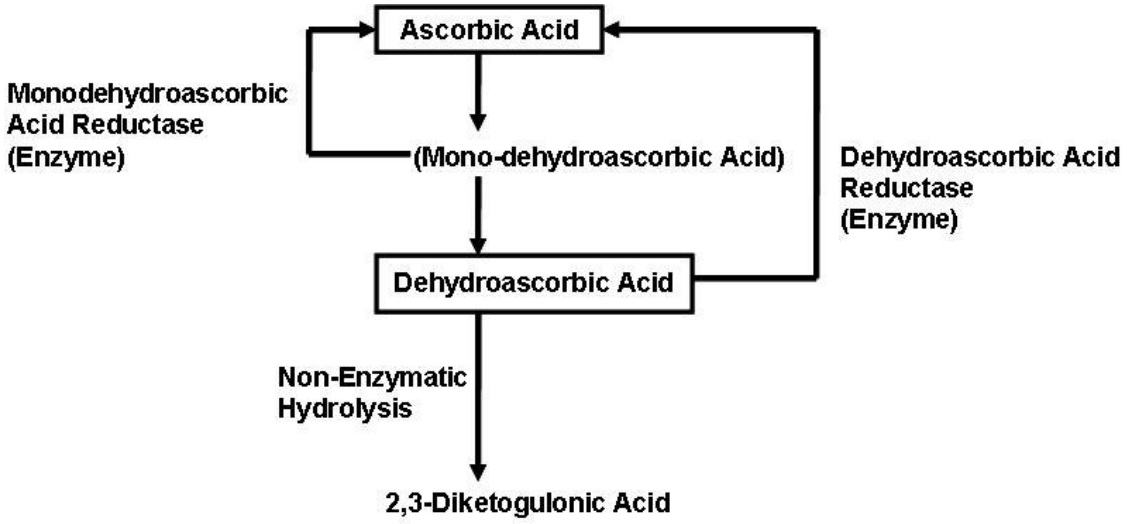

Figure 8.4 - Vitamin C redox and catabolism pathways.

The processing of chestnuts fruits inevitably produces a large amount of waste materials - shells (pericarp and integument). It is obviously of the utmost importance to create an environmentally-friendly solution that can also generate added-value co-products with economic benefits for the industries in this sector. The development of efficient and cheap processing methods for these residues could then reduce the overall fruit processing costs, increase the competitiveness of these companies and create a more sustainable agriculture. The fact that these materials are rich in biologically-active compounds, such as antioxidant polyphenols, strongly supports the development of methods to process these residues into key ingredients for functional foods (prevention of degenerative diseases, such as cardiovascular diseases and cancers) or as sources of tannins for the wine, tanning and animal feed industries. Previous works have found considerable amounts of polyphenolics and tannins in other chestnut tree tissues (wood, bark, leaf, flower, skin and fruit) (Lampire *et al*., 1998; Canas *et al*., 1999; Barreira *et al*., 2008). Clearly there is a great potential to use the integument and pericarp from the fruits, and perhaps also the burrs and other waste materials generated from chestnut tree cultivation and fruit processing.

8.2 The Future for Chestnuts

In order to optimize the industrial processing of chestnut fruits it is necessary to develop further studies that focus not only on improving the agronomy and cultivar selections (to generate higher quality raw materials i.e. the fruits), but through parallel analyses of enzymatic changes (biosynthetic and catabolic enzymes) and composition studies to define and

contextualize the changes that occur during the main processing stages thus identifying the factors that cause the nutrient losses. It is necessary to identify the best cultivars that possess optimal nutritional and processing qualities and also to evaluate changes in processing that could minimize the losses of the important nutrients. These processing changes must obviously be pragmatic and cost-effective.

On the other hand, it is clear that to prove the nutritional and health value of these fruits, future research with human cells and human intervention studies must be performed. Cell model studies, using well-characterized cancer cell and immortalized cell lines, would develop studies on the uptake, metabolism and gene effects of specific chestnut fruit components e.g. studies on the effects of the fruit phenolics and ellagitannins. It is known that gallic acid, ellagic acid and various ellagitannins have potent biological effects e.g. antioxidant and anticancer effects (Heber, 2008; Murugan *et al*., 2009; Loizzo *et al*., 2009; Bialonska *et al*., 2009). There is also evidence that the ellagitannins found in chestnut tissues have significant biological effects e.g. anticancer activity of vescalagin and castalagin (Kashiwada *et al*., 1992) and topoisomerase inhibitory effects of the acutissimins (Quideau *et al*., 2003 and 2005). Recent studies on pomegranate ellagitannins have provided information on the metabolism, uptake and effects of these tannins (Seeram *et al*., 2006; González-Sarrías *et al*., 2009; Bialonska *et al*., 2009). These studies show antioxidant and anticancer activities of some ellagic acid derivatives such as the urolithins that are formed through the metabolism of ellagic acid by the gut bacteria. The human intervention studies would involve volunteers consuming the common forms of chestnut fruits (e.g. roasted and boiled) for either a short time (e.g. an acute intervention of 100-200g in 1 day followed by blood and urine collection during 48 hours to measure various biochemical parameters and metabolites), or long-term studies (e.g. smaller amounts but regular consumption, up to 1 month) to determine health effects by measuring: *i*) biomarkers of exposure (metabolites, in the blood and urine, derived from nutrients and non-nutrients to determine the pharmacokinetics of absorption, disposition, metabolism and excretion - ADME); *ii*) biomarkers of effect e.g. changes in total antioxidant capacity of plasma and changes in gene expression (and functional protein levels) for proteins and enzymes associated with anticancer, antioxidant and immune function mechanisms.

8.3 References

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