Freeze-Dried Nutritious Chestnut Preparation Enriched With Minerals From Thermal Water

Hiba TRAD

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LIST OF ABBREVIATIONS

Atomic Absorption Spectrometry	**AAS**
Analysis Of Variance	**ANOVA**
Immortalized murine microglial cell line	**BV-2**
Castanea sativa Mill	***C. sativa***
Colony Forming Unit	**CFU**
Dulbecco's Modified Eagle Medium	**DMEM**
Dimethyl Sulfoxide	**DMSO**
German Collection of Microorganisms and Cell Cultures	**DSMZ**
Dry weight	**dw**
European Commission	**EC**
Example	**e.g.**
European Union	**EU**
Fatty Acid Methyl Esters	**FAMEs**
Food and Agriculture Organization of the United Nations	**FAO**
Functional Food Science in Europe	**FUFOSE**
Gas Chromatography with Flame Ionization Detection	**GC-FID**
Growth Inhibition by 50%	**GI_{50}**
Hank's Balanced Salt Solution	**HBSS**
High Performance Liquid Chromatography	**HPLC**
High-Performance Liquid Chromatography with Refractive Index Detection	**HPLC-RI**
High-Performance Liquid Chromatography coupled to a Fluorescence Detector	**HPLC-FL**
p-iodonitrotetrazolium chloride	**INT**
Malt Agar	**MA**
Minimum Bactericidal Concentration	**MBC**
Human Breast Carcinoma Cell line	**MCF-7**
Minimal Fungical Concentration	**MFC**
Minimum Inhibitory Concentration	**MIC**
Human Gastric Adenocarcinoma Cell line	**MKN45**
Human Melanoma Cell line	**MM127**
Monounsaturated Fatty Acids	**MUFAs**
Protected Designations of Origin	**PDO**
Polyunsaturated Fatty Acids	**PUFAs**

Roswell Park Memorial Institute	**RPMI**
Saturated Fatty Acids	**SFAs**
Sulforhodamine B	**SRB**
Human Pancreatic Adenocarcinoma Cell line	**T3M4WC**
Tryptic Soy Broth	**TSB**
Thermal Spring Waters	**TSWs**
Ultra-Fast Liquid Chromatography coupled to Photodiode Array Detection	**UFLC-PDA**
United States Dollar	**USD**
World Health Organization	**WHO**

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ABSTRACT

Chestnuts have long been a staple food of rural populations, valued for their high nutritional content, including vitamins such as vitamin C and B6, fiber, minerals and carbohydrates, all while being free of cholesterol. In Portugal, chestnuts are a widely cultivated product, leading to significant waste generation, much of which are often discarded. To address this issue, it is crucial to find sustainable methods for repurposing these by-products into high-value products.

In this context, the present work focuses on the valorisation of non- commercial chestnuts to develop a nutritional preparation using the freeze-dried technique. This method involves a dehydration process that removes water from food, preserves the nutritional quality of chestnut, and to extends their shelf life. Additionally, the preparation will be enriched with beneficial minerals derived from the thermal water, further enhancing its nutritional value. This approach aligns with the principals of sustainable food production by reducing waste and contributing to the circular economy.

Four Portuguese chestnut varieties (Amarelal, Boa Ventura, Judia and Longal) were characterized regarding their nutritional composition, chemical profile, mineral composition and bioactive properties. Nutritional evaluation was caried out by determining the fat, protein, ash, carbohydrates and energy value. Chemical characterization was performed resorting to different chromatographic techniques, namely High-Performance Liquid Chromatography with refractive index detection (HPLC-RI) for free sugars, High-Performance Liquid Chromatography coupled to fluorescence detection (HPLC-FL) for tocopherols, Ultra-Fast Liquid Chromatography coupled to photodiode array detection (UFLC-PDA) for organic acids, and Gas Chromatography with flame ionization detection (GC-FID) for fatty acid profiling. Mineral composition and bioactive properties, including antimicrobial activity and cytotoxicity, were also evaluated.

The results showed that chestnuts presented relevant and functional properties, with carbohydrates representing the major macronutrient and unsaturated fatty acids being predominant in the lipidic fraction. Sucrose was the major free sugar determined, while regarding organic acids, tocopherols and essential minerals presented themselves in considerable amounts. All varieties exhibited moderated to low antimicrobial potential

and low cytotoxicity, supporting their potential application in food products. The incorporation of thermal water contributed to the mineral enrichment of the freeze-dried formulation while maintaining their nutritional quality.

The final product, rich in vitamins, minerals, and bioactive compounds, demonstrated promising potential as a functional food ingredient for applications such as health drinks, fortified foods, or dietary supplements. Overall, this work highlights the feasibility of valorizing non-commercial chestnuts through sustainable technological approaches, contributing to waste reduction, food innovation, and the development of value-added products with potential industrial applications.

Key words: chestnut by- products, freeze drying, functional food, minerals, thermal water, valorization, waste.

RESUMO

As castanhas há muito que são um alimento essencial das populações rurais, valorizadas pelo seu elevado teor nutricional, incluindo vitaminas como a vitamina C e B6, fibra, minerais e hidratos de carbono, sendo ainda isentas de colesterol. Em Portugal, as castanhas são um produto amplamente cultivado, resultando numa geração significativa de resíduos, muitos dos quais são frequentemente descartados. Para enfrentar este problema, é crucial encontrar métodos sustentáveis para reaproveitar estes subprodutos e transformá-los em produtos de alto valor.

Neste contexto, o presente trabalho investigação centrou-se na valorização de castanhas não comerciais para desenvolver uma preparação nutricional utilizando a técnica de liofilização. Este método envolveu um processo de desidratação que remove a água dos alimentos, preserva a qualidade nutricional da castanha e prolonga o seu prazo de validade. Além disso, a preparação foi enriquecida com minerais benéficos derivados da água termal, aumentando ainda mais o seu valor nutricional. Esta abordagem está alinhada com os princípios da produção alimentar sustentável, reduzindo o desperdício e contribuindo para a economia circular.

Foram caracterizadas quatro variedades portuguesas de castanha (Amarelal, Boa Ventura, Judia e Longal) quanto à sua composição nutricional, perfil químico, composição mineral e propriedades bioativas. A avaliação nutricional foi realizada através da determinação do teor de gordura, proteína, cinzas, hidratos de carbono e valor energético. A caracterização química foi realizada utilizando diferentes técnicas cromatográficas, nomeadamente Cromatografia Líquida de Alta Eficiência com deteção por índice de refração (HPLC-RI) para açúcares livres, Cromatografia Líquida de Alta Eficiência acoplada à deteção por fluorescência (HPLC-FL) para tocoferóis, Cromatografia Líquida Ultrarrápida acoplada à deteção por arranjo de fotodíodos (UFLC-PDA) para ácidos orgânicos e Cromatografia Gasosa com deteção por ionização de chama (GC-FID) para perfil de ácidos gordos. A composição mineral e as propriedades bioativas, incluindo a atividade antimicrobiana e a citotoxicidade, também foram avaliadas.

Os resultados mostraram que as castanhas apresentaram propriedades relevantes e funcionais, sendo que os hidratos de carbono representam o principal macronutriente e os

ácidos gordos insaturados foram predominantes na fração lipídica. A sacarose foi o principal açúcar livre determinado, enquanto, em relação aos ácidos orgânicos, os tocoferóis e os minerais essenciais se apresentaram em quantidades consideráveis. Todas as variedades exibiram um potencial antimicrobiano moderado abaixo e uma baixa citotoxicidade, o que corrobora o seu potencial de aplicação em produtos alimentares. A incorporação de água termal contribuiu para o enriquecimento mineral da formulação liofilizada, mantendo a sua qualidade nutricional.

O produto final, rico em vitaminas, minerais e compostos bioativos, demonstrou um potencial promissor como ingrediente de alimentos funcionais para aplicações como bebidas funcionais, alimentos fortificados ou suplementos alimentares. De um modo geral, este trabalho destaca a viabilidade de valorizar as castanhas não comerciais através de abordagens tecnológicas sustentáveis, contribuindo para a redução de resíduos, inovação alimentar e desenvolvimento de produtos de valor acrescentado com potencial para aplicações industriais.

Palavras-chave: subproduto de castanhas, liofilização, alimento funcional, minerais, água termal, valorização, desperdício

1. Introduction

Chestnuts trees are versatile species primarily distributed across the Northern Hemisphere, valued for their dual-purpose yield of fruit and wood. They play a crucial role in both the economic and ecological systems **(Braga et al., 2023).**

The chestnut belongs to the Fagaceae family, and the Castaneoidiae subfamily. It is classified under the *Castanea* genus, a name derived from "Kastah." meaning "dried fruit" in Eastern Asia. This genus includes *Castanea sativa* and 12 other diploid species(**Braga et al., 2023; Santos et al., 2022).**

Different *Castanea* species are distributed across various regions of the world, including China (*Castanea mollissima* BL. and *Castanea seguinii* Dode.), Japan (*Castanea crenata* Sieb. & Zucc.), North America (*Castanea dentata* (Marsh.) Brokh), and Europe (*Castanea sativa* Mill.) Among these species, *C. sativa* Mill. is recognized as one of the significant, particularly as a traditional fruit crop in the Mediterranean regions of Europe **(Santos et al., 2022).**

In recent years, the global cultivation and productivity of chestnut have been increasing. However, Europe, chestnut production has remained relatively stable, with reported yields of 302440 tons recorded in 2022 and 312370 tons in 2023 **(FAOSTAT, 2024)**.

2. Literature Review

2.1. Chestnut cultivation in Portugal

The chestnut tree (*Castanea sativa* Mill.) was introduced and distributed in Portugal, as well as in other European countries, by the Romans during their period of colonization **(Braga et al., 2023)**.

C. sativa Mill. is the most cultivated chestnut species in Portugal and throughout Europe. It is considered one of the oldest and most traditional edible fruits in Portugal and was historically used as extensively as potatoes and other tubers **(Borges et al., 2008).**

In 2022, Portugal ranked eighth in global chestnut production, with a total output of 22,670 tons, a significant decrease from 37,720 tons in 2021**(FAOSTAT, 2024)**. This decline has been attributed to various factors, including climate changes and both biotic

and abiotic stresses, which are considered major threats to the sustainability of chestnut cultivation in Portugal **(Braga et al., 2023).**

The Trás-os-Montes region is the primary chestnut producing area in Portugal, accounting for approximately 85% of the country's total production **(Santos et al., 2023).**Within this region, chestnut cultivation is concentrated in Bragança and Vila Real, contributing 59% and 19%, respectively, to national production. The Bragança region alone has 12,500 hectares dedicated to chestnut cultivation. These areas provide optimal conditions for *C. sativa* growth, characterized by elevations above 500 meters and low winter temperatures **(Freitas et al., 2022; B. Ribeiro et al., 2007).**

In Portugal, chestnut trees play a vital role in rural economies, serving a dual purpose as they are cultivated for their fruit and for their high-quality wood **(Braga et al., 2023).**

2.2. Chestnut Varieties in Portugal

To preserve the identity and biodiversity of Portuguese chestnuts and improve their cultivation, Protected Designations of Origin (PDO) were established under (EU Regulation no. 1151/2012). There were 4 PDO for chestnuts: ("Castanha da Padrela" and "Castanha da Terra Fria") in Trás-os-Montes, ("Castanha de Marvão-Portalegre") in the region of Portalegre and the fourth one is ("Castanha dos Soutos da Lapa") in Beira Alta. Each PDO represents unique historical backgrounds, predominant varieties, and specific production areas **(Braga et al., 2023; M Ribeiro et al., 2020)**.

Portugal cultivates approximately 23 cultivars of *C. sativa,* with the most important being Boa Ventura, Judia, Longal, and Martaínha. Other cultivated varieties include Amarelal, Benfeita, and Negral. The production of Amarelal, Aveleira, Boa Ventura, Côta, Judia, Lamela, Longal, Martaínha, Negral, and Trigueira, which are classified under the "Castanha da Terra Fria PDO", is concentrated in municipalities such as Alfândega da Fé, Bragança, Chaves, Macedo de Cavaleiros, Mirandela, Valpaços, Vimioso, and Vinhais **(Braga et al., 2023).**

Some varieties of chestnut from Braganca are shown in Figure 1.

Figure 1: The Portuguese's chestnuts varieties.

These varieties differ in size, texture, flavors, sweetness. For example, "Judia" is the largest variety, while "Longal" is known for being easy to peel and preserve, with a very pleasant flavour **(Freitas et al., 2022).**

2.3. Nutritional value of chestnuts

Portuguese chestnuts are highly valued both nationally and internationally for their exceptional quality**(Braga et al., 2023).** Due to their unique nutritional composition and sensory characteristics, they have played an essential role in human nutrition since ancient times and are widely recognized for their potential health benefits**(Santos et al., 2023)**.

Additionally ,they are naturally gluten-free, making them an ideal food for individuals with gluten sensitivity and intolerance **(Gomes-Laranjo et al., 2025).**

Chestnuts contain 40 - 64 g of water per100 g fresh weight **(Santos et al., 2022).** They are also rich in carbohydrates, primarily starch and sugars such as sucrose, and provide high biological value protein, dietary fiber, and essential vitamins (B6, vitamin C and folic acid). In addition, they contain key minerals, including potassium, phosphorous,.and magnesium, as well as lipids and micronutrients (**Table 1) (Braga et al., 2023; Santos et al., 2022).**

Table 1: Nutritional value of 100 g of raw European chestnut **(Adapted by Santos et al., 2022).**

Nutrients	***Values***
Water	52
Protein	1,63
Total lipid (fat)	1,25
Carbohydrates	44,2
Vitamins (mg)	
Vitamin A	26
Vitamin C	40,2
Niacin	1,1
Electrolytes (mg)	
Sodium	2
Potassium	484
Minerals (mg)	
Calcium	19
Iron	0.94
Magnesium	30
Manganese	0,336
Phosphorus	38
Zinc	0,49

Furthermore, chestnuts are an excellent source of antioxidants including L-ascorbic acid, carotenoids, and phenolic compounds such as gallic and ellagic acids. They also have a low- fat content, with high concentrations of unsaturated fatty acids (linoleic and oleic acid), making them a nutritionally valuable food **(Foucher et al., 2022; Santos et al., 2022).**

Studies on Portuguese chestnut varieties (Judia and Longal) have identified seven organic acids: oxalic, cis-aconitic, citric, ascorbic, malic, quinic and fumaric acids. These compounds exhibit strong antioxidant properties and may provide protection against various diseases **(Gonçalves et al., 2010)**.

2.4. Waste generated by chestnut cultivation and the growing problem of waste disposal

Every year, thousands of tons of agricultural waste are generated across Europe without efficient utilization by producers or agri-food-industry sector. This leads to increased economic and environmental costs associated with waste management **(Aires et al., 2016)**. The agro-industrial sector is a major contributor to waste generation, negatively impacting both the environment and economy **(Amato Davide et al., 2021; Chiocchio et al., 2020**). In this context, the valorisation of crop waste plays a crucial role in the circular economy. Recovering valuable bioactive compounds from industrial by-products provides a sustainable solution for waste reduction and promote efficient resource recycling **(Conidi et al., 2022)**.

Industries specializing in chestnut processing generate approximately 7000 tons of processed chestnut products annually, resulting in substantial waste production **(Barletta et al., 2025).**

Notably, waste from chestnut industry, particularly non-commercial chestnuts that do not meet the size or shape requirements for sale, represents an attractive resource due to its high bioactive compound content, and interesting nutritional profile. This by-products have potential applications in various industries, including, pharmaceutical, nutraceutical or cosmetic sectors **(Amato Davide et al., 2021).** Additionally, chestnut by -products can be processed into functional foods, nutraceuticals, or fortified beverages, enhancing their commercial value **(de Ancos et al., 2018).**Proper management of chestnut

waste is essential for improving the economic sustainability and competitiveness of the chestnut supply chain, minimizing its environmental impact **(Donno et al., 2024).**

2.5. Sustainability in Food Production

Freeze-drying, also known as cryodesiccation or lyophilization, is a dehydration process initially employed in the Western Europe for the food industry. Over time, this technology has been adopted by several countries, including the United States, England, France, Japan, just to name a few **(Dabbour et al., 2022; Liu et al., 2022).**

The primary goal of freeze-drying is to preserve the nutrients and bioactive substances in raw materials while maintaining their original characteristics such as colour, fragrance, flavour, taste, among others **(Castañeda-Saucedo et al., 2014; Liu et al., 2022).**

Additionally, freeze-drying is utilized to convert biowaste products into valuable materials, such as powders, or even, microencapsulate bioactive compounds. These can be further used to enhance the quality of industrially processed products **(Coşkun et al., 2024).**

2.6. Freeze-drying process

Freeze-drying process consists of three major stages, as illustrated in Figure 2: Freezing, Primary Drying (Sublimation Phase), and Secondary Drying (Desorption Phase). In the first stage (Freezing), solid products (e.g., fruits, vegetables) and liquid products must be frozen before freeze-drying. During freezing, the liquid water in food is transformed into ice crystals, varying in size, depending on the cooling process. The freezing temperature varies based on the composition of the sample, specific temperatures are required taking into consideration the composition of the sample regarding the presence of protein, fat, and other components. In large-scale food industry applications, raw materials are typically frozen at temperatures between -20°C and -40°C for 1 to 4 hours using the air blast freezing method. Water crystallization limits chemical, biochemical, and microbiological reactions thereby preserving the material **(Coşkun et al., 2024; Ma et al., 2023; Nowak & Jakubczyk, 2020).**

In the second step (Primary Drying, sublimation stage), the ice crystals sublimate directly into the vapor phase without becoming liquid. This sublimation reaction occurs

under low pressure and temperature, ensuring the products structural and nutritional integrity **(Coşkun et al., 2024).**

Finally, the third step (Secondary Drying, desorption stage), concludes the freeze-drying process. In this stage, the products water content is reduced to below 5% **(Ma et al., 2023)**. The final moisture content determines stability and storage life of the product. Excessive moisture can reduce shelf life, while too little moisture can damage the active compounds. Shorter drying times are associated with higher product quality **(Coşkun et al., 2024; Nowak & Jakubczyk, 2020).**

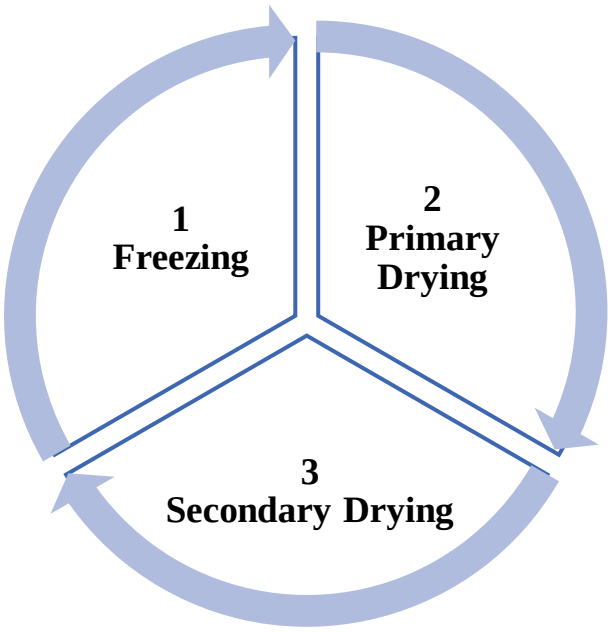


Figure 2: Freeze-drying Stages **(Adapted by Coşkun et al., 2024).**

Several parameters influence the freeze-drying process, including temperature and pressure which play key roles in preserving bioactive content and products flavor, among other properties **(Coşkun et al., 2024).**

2.6.1. Freeze-drying method in fruit and nut preservation

Freeze-drying is widely used in both the food and pharmaceutical industries. It is regarded as one of the most effective dehydration techniques for products **(Castañeda-Saucedo et al., 2014; Waghmare et al., 2022).**

The applications of freeze-drying in the food sector are illustrated in Figure 3

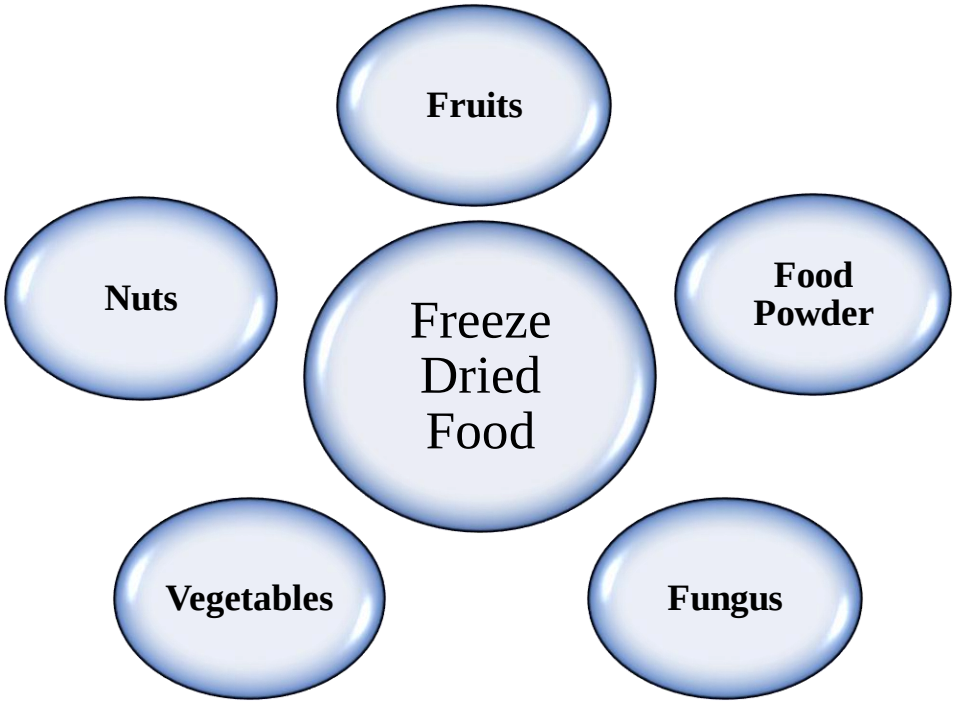


Figure 3: Applications of freeze-drying in food industry **(Adapted by Liu et al., 2022).**

2.6.2. Applications in Fruit Preservation

Fruits are naturally rich in vitamins and amino acids. However, their high moisture content and delicate texture present storage challenges. To address this issue, freeze-drying has been extensively studied for its ability to preserve fruit's quality **(Liu et al., 2022; Ma et al., 2023)**. This technique provides several advantages, such as extended shelf life, reducing weight and facilitating easier transportation to distant markets **(Castañeda-Saucedo et al., 2014).** For example, freeze-drying technique can preserve the color of the strawberries by preventing browning reaction; this technique applied in mangoes showed that could effectively minimize the loss of phenolic content and ascorbic acid; on white mulberries freeze-drying also proved that can preserve the fruits' quality including its bioactive ingredients and physical properties **(Coşkun et al., 2024; Liu et al., 2022**)**.**

2.6.3. Applications in nut Preservation

The freeze-drying technique is also largely applied to nuts, particularly chestnuts. Studies performed in '*Castanea Mollissima'* (Chinese chestnut) have demonstrated that this technique improves nut quality, extends shelf life, and reduces post-harvest losses, especially when combined with coating technologies **(Gounga et al., 2008).**

2.6.4. Benefits of freeze-drying method

Freeze-drying, has been increasingly used in recent years to retain high-valued materials and achieve superior product quality compared with other drying methods **(Harguindeguy & Fissore, 2020)**. The major benefits include minimal shrinkage during drying, easy rehydration upon adding water, a well-defined porous structure, lighter weight, and sterile and easy handling **(Ciurzynska & Lenart, 2011; Liu et al., 2022).** Due to its controlled conditions, it preserves volatile compounds, that contribute to flavor and aroma, antioxidants, vitamins, minerals, and, maintaining protein integrity, preventing oxidative degradation and Maillard reactions**(Dabbour et al., 2022; Silva et al., 2017).** As a result, freeze-drying products retain bioactive properties beneficial to human health **(Coşkun et al., 2024).**

2.6.5. Comparison between freeze-drying and other preservation methods

Due to its ability to dehydrate food products via sublimation, freeze-drying is more effective than other drying methods, such as sun drying and oven drying, in preserving nutrients, and other important properties. Studies have shown that freeze drying method is better retaining total phenolic content, flavonoids, antioxidant activities, and vitamin C **(Sapkota et al., 2023),** minimizes the degradation of bioactive components when compared to other methods **(N. C. Silva et al., 2017),** preserves higher levels of chlorophyll and total saponins compared to vacuum drying and hot-air drying **(Liu et al., 2022)**.This technique also enhances the retention of anthocyanins, as observed in Cornelian cherry **(Coşkun et al., 2024).**

Overall, freeze-drying outperforms alternative methods in preserving nutritional and functional properties, making it an optimal choice for high-quality food preservation.

2.6.6. Challenges and Limitations

Despite its advantages, freeze-drying, is one of the most expensive dehydration techniques due to its high energy consumption and operational costs. Compared to air-drying, lyophilization costs are estimated to be 4–8 times higher. The process requires low temperatures and pressures, leading to increased energy usage. Studies indicate that the energy required to remove 1 kg of water via freeze-drying is approximately double that of conventional drying methods **(Ciurzynska & Lenart, 2011)**.

The energy consumption for freeze drying operation is presented in Figure 4**.**

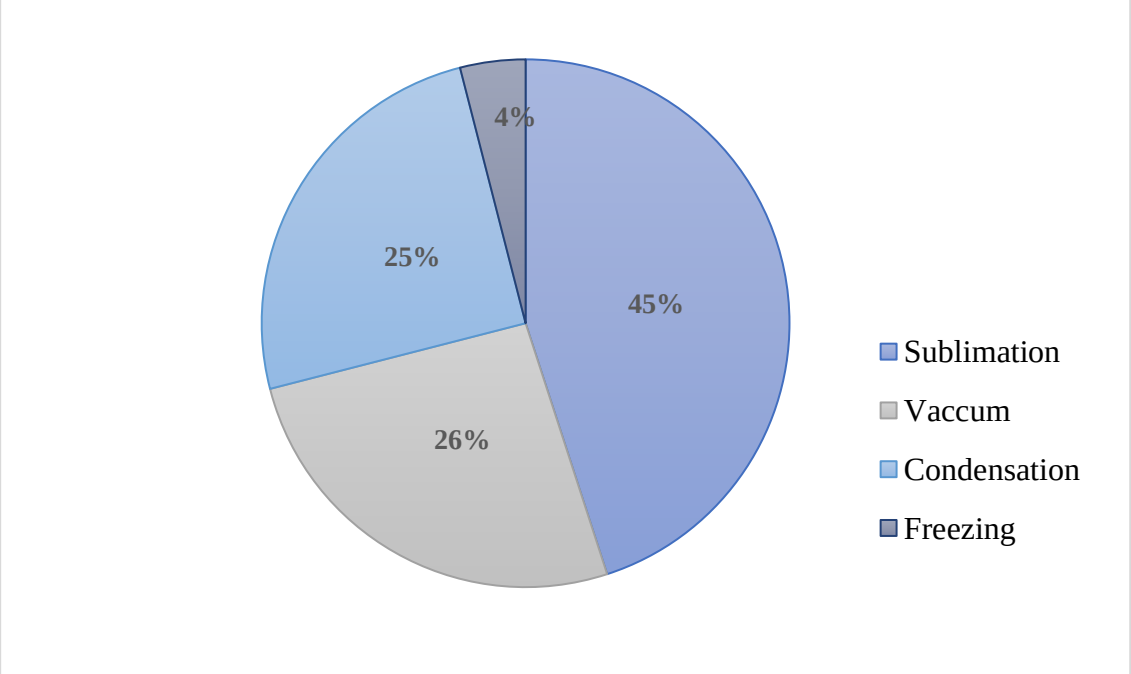


Figure 4: Energy consumption for freeze-drying process **(Adapted by Ratti, 2001).**

To address this issue, alternative methods such as vacuum drying, and hot air drying have been explored to retain bioactive compounds **(de Ancos et al., 2018)**. Each technique has specific advantages and disadvantages, and the choice depends on the product requirements. Table 2 provides an overview of different drying techniques used in the food industry.

Table 2: Advantages and limitations of different drying techniques ***(*Adapted by Belwal et al., 2022).**

Drying Technique	*Advantages*	*Disadvantages*
Freeze-drying	Produce High quality of products.	Prolonged drying time. Elevated costs of energy and investment.
Hot air drying	Easy and economical drying method.	Elevated thermal energy requirements.
Spray drying	Fast technique drying, Applicable for various product.	Maintenance Problems.
Vacuum drying	Ideal drying method for heat sensitive materials.	Efficiency reduced.
Sun drying	Limited Costs compared to other methods.	High temperature drying process needed. Requires high level of human labor.

2.7. Applications of Functional Foods

Functional foods do not have a universally accepted definition. However, according to the **European Commission's (EC)** initiatives, such as the Concerted Action Concerted Action on Functional Food Science in Europe **(FUFOSE)**, functional foods are defined as those that provide health benefits beyond basic nutrition. These foods are designed not only to satisfy hunger and supply essential nutrients but also to promote overall well-being and reduce the risk of diseases. They are intended to be consumed as part of regular healthy diet rather than in pill or a capsule form **(European Commission. Directorate-General for Research, 2010; Menrad, 2003).**

Functional foods can be naturally occurring or developed by processing or agro-biotechnological methods. Regardless of their origin, their safety must be always ensured (**Kahl et al., 2012; Pinela et al., 2023**).

There are five main approaches to obtaining functional food products:

- Elimination of toxic components that negatively impact consumption (*e.g.*, gluten allergic protein in cereals).

- ❖ Enrichment by increasing the concentration of a naturally occurring constituent in food to enhance its nutritional quality.
- ❖ Incorporation of beneficial compounds not typically present in most foods, such as non-vitamin antioxidants.
- ❖ Substitution of macronutrients, such as replacing lipids with components that have demonstrated positive health effects.
- ❖ Enhancement of the bioavailability or stability of specific compounds to increase their functional effects or reduce the disease-risk potential of the food **(Henry, 2010).**

2.7.1. Classification of functional foods

Functional foods can be categorized into three major classes: conventional foods, modified foods, and synthesized food ingredients. Conventional functional foods are naturally rich in essential nutrients such as vitamins, minerals, and antioxidants, providing health benefits beyond basic nutrition. These foods do not require any modifications or enhancements, as their bioactive components occur naturally. Examples include vitamin E-enriched vegetable oils, fruits, vegetables, whole grains, nuts, and seeds, which contribute to improved health by supporting immune function, reducing oxidative stress, and lowering the risk of chronic diseases.

Modified functional foods, on the other hand, are foods that have been fortified or enriched with bioactive compounds to enhance their nutritional value and potential health benefits. These modifications may involve the addition of vitamins, minerals, probiotics, omega-3 fatty acids, or phytochemicals to promote overall well-being and disease prevention. A common example is yogurt enriched with probiotics, which supports gut health and improves digestion. Lastly, synthesized food ingredient includes specially designed compounds, such as certain carbohydrates, that exhibit functional properties. These ingredients, like prebiotics, are formulated to selectively stimulate the growth and activity of beneficial gut bacteria, enhancing gut microbiota balance and overall digestive health. The major classes of functional foods are presented in the Table 3.

Table 3: Major Categories of functional foods ***(*Adapted by Ajmera, 2024; European Commission. Directorate-General for Research, 2010; Henry, 2010; Vlaicu et al., 2023).**

Categories	*Definitions*	*Examples*
Conventional foods	They are naturally rich in essential nutrients such as vitamins, minerals, and antioxidants.	Vitamin E-enriched vegetable oils.
Modified food	Fortified with bioactive elements like vitamins, minerals, probiotics, omega-3 fatty acids, and phytochemicals	Yogurt with added probiotics.
Synthesized food ingredients	Includes specialized carbohydrates designed to have probiotic effect	

2.7.2. Benefits of functional foods

Functional foods are known for their ability to decrease the risk of chronic diseases and to control current health issues, such as heart disease, diabetes, and hypertension. Additionally, they have an essential role in reinforcing the immune system, enhancing the body's defense mechanisms against infections and diseases due to their richness in bioactive components including omega-3 fatty acids, antioxidants, polyphenols, essential minerals, and vitamins which have a several health benefits **(Vlaicu et al., 2023)**.For example nuts are nutrient-rich foods containing several bioactive compounds such as phenolic and flavonoid which are beneficial for cardiovascular health. The studies from International Tree Nut Council Nutrition Research and Education Foundation have shown that the introduce of nuts into the diet can minimize the risk of heart disease **(Glenn et al., 2023; Kaur & Das, 2011).**

The Table 4 presents some examples of ingredient introduced to obtain functional foods and their benefits for health.

Table 4: Functional ingredients, sources, and benefits (**Adapted by Kaur & Das, 2011).**

Bioactive Elements	*Description*	*Health benefits*	*Functional food*
Lycopene	Natural carotenoids present in red colored fruits and vegetable (example tomato).	Antioxidant Protect the oxidative stress, the hypertension, the atherosclerosis, and the diabetes	Dry fermented sausage fortified with lycopene.
Resveratrol	Polyphenol present in grapes, nuts	Cardioprotective Anticancer, Anti-inflammatory Antioxidant Modulator of ipid metabolism	Functional peanut with an enhanced resveratrol content.
Docosahexaenoic acid (DHA)	ω-3 fatty acid present in seafood oil (example: fish, marine algae)	Antioxidant Antiviral Anticancer agent Antihypertensive	Pasta with edible Japanese seaweed, wakame (*Undaria pinnatifida*)

2.8. Analysis of market trends for functional foods targeting malnutrition and consumer health

The market value of functional foods and beverages, around the world, was estimated in 2021 at USD 258 billion. This value is projected to attend around USD 530 billion in 2028 **(Vlaicu et al., 2023).** The major sector of the food market where functional food products have been predominantly introduced include dairy, bakery, confectionery, soft drinks, and baby foods **(Kaur & Das, 2011).**

The European functional food market, it has been expanding due to increasing consumer awareness of self-care, an aging population, and rising health-care costs. High product quality has contributed to the market's success. Consumers increasing recognize the impact of diet on health and prefer functional foods over synthetic supplements. They tend to choose natural options based on taste preferences **(Kahl et al., 2012; Sun-Waterhouse, 2011)**.

Functional foods bridge the gap between conventional nutrition and therapeutic benefits. They resemble traditional foods in nutritional value and appearance but provide additional health benefits when consumed regularly **(Pinela et al., 2023; Vlaicu et al., 2023).**

2.9. Nutritional Enrichment Strategies

2.9.1. The importance of nutritional enrichment and the role of minerals in functional foods

The processing of functional foods presents a significant scientific challenge due to factors that can lead to the degradation of micronutrients and bioactive compounds, thereby affecting physicochemical parameters. To address these challenges, various technological advancements have been introduced in the food sector. Several techniques are employed to enhance the bioactive compounds content, thereby maximizing the potential of functional foods. These techniques include technological innovations, advanced digital methods, and improvements in nutrition **(Pinela et al., 2023; Vlaicu et al., 2023)**.

These methods improve the bioavailability of bioactive compounds, such as minerals, which play essential roles in our body, ranging from building strong bones to transmitting nerve impulses. Of the sixteen essential minerals, eleven are required in trace amounts and are naturally present in food and drinking water. Iodine *(I),* iron *(Fe),* zinc *(Zn),* calcium (*Ca)* and selenium *(Se),* are often present in limited quantities in many foods. An insufficiency in these bioactive constituents can lead to common disorders problem and disease symptoms. Mineral deficiencies are particularly prevalent in developing countries due to limited access to fresh food and drinking water. To enhance their absorption and bioavailability, it is essential to implement safe food fortification and processing techniques (**Gharibzahedi & Jafari, 2017; Gómez-Galera et al., 2010; Vlaicu et al., 2023)**.

2.9.2. Importance of minerals in thermal water

Thermal spring waters (TSWs), or “mineral–medicinal waters”, are known for their rich mineral content and therapeutic properties. Their composition includes chloride, bicarbonate, sulfate, sulfide, and carbon-dioxide-rich compounds. Additionally, they may contain soluble minerals such as Magnesium (Mg^{+2}), Calcium (Ca^{+2}), Sodium (Na^{+}),

Silicates (SiO_2), as well as trace mineral elements such as manganese, selenium, zinc, and boron (B) **(Mourelle et al., 2024)**(**Figure 5**).

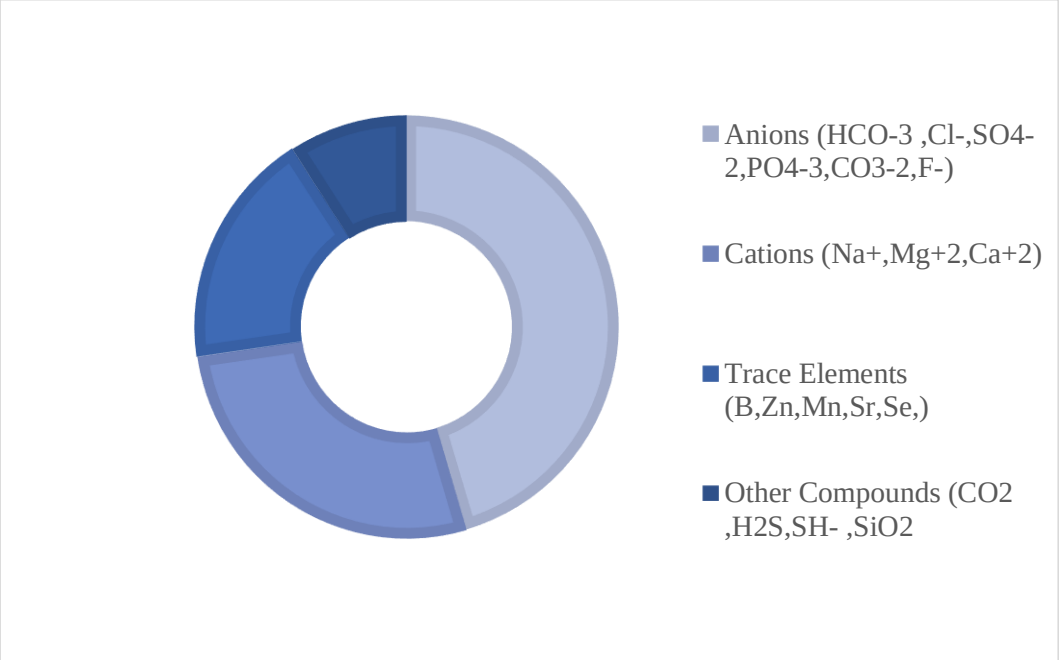


Figure 5 : Composition of Thermal water (**Adapted by Mourelle et al., 2024*).***

Each mineral or trace element offers specific health benefits. For instance, selenium acts as a cofactor for approximately 50 enzymes, including glutathione peroxidase, an essential antioxidant enzyme. It also plays a role in thyroid hormone metabolism by aiding in removal of mineral ions from other proteins (e.g., thyroid hormone deiodinases). Selenium is considerate an antioxidant with potential health benefits, such as reducing the risk of cancer and cardiovascular diseases **(Gómez-Galera et al., 2010).**

2.9.3. Comparison of different methods for incorporating minerals into functional foods

The production of functional food integrates various processing methods, ranging from traditional agricultural practices to advanced technological innovations.

Techniques such as extraction, encapsulation, fortification, and enrichment, are used to enhance bioactive compound concentration and improve their absorption in the human body **(Vlaicu et al., 2023)** .

When addressing minerals deficiencies, several strategies (**Table 5**) have been implemented. Fortification and enrichment are often used interchangeably, involving addition of bioactive compounds (e.g., vitamins and minerals) to food to enhance its nutritional properties **(Vettorazzi et al., 2020)**.

According to the World Health Organization (WHO), fortification is one of the most effective strategies for improving food quality while reducing health risks. This process mitigates specific mineral deficiencies by incorporating nutrients into food during preparation, processing, and agricultural practices **(Manzoor et al., 2024; World Health Organization, 2024).** Fortification aims to enhance nutrient absorption and bioavailability while positively impacting consumer health **(Gharibzahedi & Jafari, 2017)**.

Biofortification is another effective approach that enhances the nutrient content and quality of specific crops. It provides a cost-effective and sustainable solution to malnutrition, especially in developing countries, by focusing on staple foods consumed daily **(Gharibzahedi & Jafari, 2017).** Compared to traditional fortification, biofortification is more sustainable, as biofortified crops can be cultivated for years without requiring ongoing intervention. Additionally, bio-fortified foods provide more bioavailable nutrients and pose a lower risk of overdose than fortified foods **(Manzoor et al., 2024)**.

Nanoencapsulation is an advanced technique that utilizes nano-sized particles (less than 1000 nm) to encapsulate and deliver bioactive compounds such as vitamins, minerals, and antioxidants. This technique enhances the physicochemical stability, water solubility, bioaccessibility and bioavailability of these compounds, thereby improving their health benefits **(Gharibzahedi & Jafari, 2017; Vlaicu et al., 2023)**.

Table 5: Methods and processing operations used to incorporate minerals. **(Adapted by Gharibzahedi & Jafari, 2017; Manzoor et al., 2024).**

Methods	*Advantages*	*Inconvenient*
Fortification	The most effective, and efficient technique for delivering essential micronutrients for large populations. Enhancing the nutritional quality of the food supply while decreasing health risks. Food fortification is a cost-effective approach.	Continuous process Provide less bioavailable nutrients compared to other methods.
Biofortification	Excellent approach to improve the nutrient content and quality of a specific crop. It's a cost-effective and sustainable solution to combat malnutrition. Provide more bioavailable nutrients to the human body and have a lower risk of overdose Biofortified crops can be used many years.	
Nanoencapsulation	Advanced technique Enhancing the physicochemical stability, water-solubility, bioaccessibility and bioavailability of the bioactive compounds.	

3. Objectives

Chestnut by-products have gained significant interest due to their low cost, high content of bioactive compounds, and valuable nutritional profile. Their utilization presents a promising approach not only for enhancing economic development but also for minimizing waste disposal costs, thereby contributing to sustainable food production.

The primary objective of this study is to valorize non-commercial chestnuts by developing a freeze-dried nutritional preparation derived from chestnuts and enriched with minerals from thermal water.

Specific Objectives

- ✓ Chestnut varieties samples characterization: conduct by an exhaustive chemical, bioactive and nutritional analysis.
- ✓ Product Specification: definition of key characteristics and requirements for the developed product.
- ✓ Freeze-drying process: optimize the freeze-drying technique to dehydrate the developed product.
- ✓ Product Characterization: Monitoring the chemical, and nutritional parameters of the developed product.

Figure 6: Schematic representation of the experimental workflow and the objectives of the present study.

4. Materials and methods

4.1. Plant material treatment

The four varieties of C. *sativa*, Amarelal, Boa Ventura, Judia and Longal were collected in November 2024 in Bragança, Portugal, coinciding with the usual chestnut harvest season, after collection, the fruits underwent an initial post-harvest procedure consisting of manual cleaning and sorting, during which damaged or deteriorated specimens were removed to avoid compromising the remaining material. The selected chestnuts were then carefully peeled (**Figure 7)** and cut into small pieces to facilitate subsequent processing.

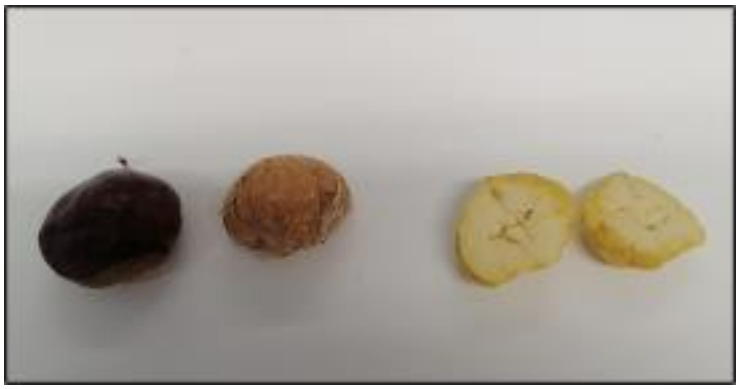


Figure 7: Chestnut before and after peeling.

The prepared material was placed in dehydrator and dried under controlled conditions until all moisture had been completely removed. Once dried, the samples were ground using a laboratory grinder (Retsch, ZM200) to obtain a homogeneous powder. The resulting material was then stored in tightly sealed bags, protected from light and humidity until further analysis.

The nutritional composition of the chestnut samples was determined according to the official analytical methods currently in force of the AOAC INTERNATIONAL **(AOAC INTERNATIONAL, 2023)**, ensuring the accuracy, reliability and comparability of the obtained results. In addition to the proximate composition analysis, the chemical characterization of the samples was carried out using different chromatographic techniques, including the determination of individual compounds. The analytical procedures and chromatographic conditions employed for each class of compounds are described in detail in their respective sections.

4.2. Characterization of the different chestnut varieties

4.2.1. Evaluation of nutritional value

4.2.1.1. Fat content

The fat content of the samples was determine using the Soxhlet extraction method, one of the most established procedures for quantitative lipid analysis. Approximately 3g of each powdered chestnut sample were placed in cellulose extraction cartridges (Ø 26 x 60 mm), and 40-50 mL of petroleum ether were added to the Soxhlet extraction flasks (Ø 51 x 59 mm).

The extraction process follows three consecutive phases. In the boiling phase, the solvent is heated to its boiling point, generating vapors that rise to the condenser, where they liquify and flow back onto the sample. During this step, the sample remains submerged, allowing the condensed solvent to dissolve the lipids through continuous immersion and reflux. In the rinsing phase, the level of condensed solvent decreases, and the sample is no longer submerged, the lipid extraction proceeds exclusively through solvent dripping by reflux. Finally, the recovery phase, the solvent is siphoned back into the boiling flask for collection and potential reuse, while the extracted fat accumulates in the lower section of the flask.

After approximately one hour of extraction, the solvent was evaporated, and the lipid fraction was transferred to pre-weighed tubes. These were left to dry as room temperature to ensure complete removal of the residual extraction solvent.

The fat content was determined gravimetrically by calculating the difference between the mass after extraction and their initial mass, relative to the mass of the sample:

$$\textbf{Fat content (g)} = \frac{\mathbf{m_{tube+fat} - m_{tube}}}{\mathbf{m_{initial\ sample}}}$$

4.2.1.2. Ash content

The ash content of the samples was determined using a muffle furnace. Prior the analysis, the crucibles were cleaned, oven-dried to eliminate residual moisture, and subsequently weighed in order to record their initial mass. Approximately 500 mg of each sample were then placed in the crucibles, which were introduced into the muffle furnace and incinerated at 500°C for 5 hours.

After cooling in a desiccator, the crucibles containing the remaining inorganic residue (ash) were weighed again **(Figure 8)**. The ash content was calculated gravimetrically by determining the difference between the mass of the crucible after incineration and its initial empty mass, relative to the initial mass of the sample.

Figure 8: Crucibles with ash.

4.2.1.3. Crude protein

The protein content of the samples was determined using the Kjeldahl method, which quantifies total nitrogen as an indirect measure of protein. Approximately 500 mg of each powered sample were weighed into digestion tubes, to which 15 mL of sulfuric acid (H_2SO_4) and a catalyst were added to accelerate the reaction. The tubes were then placed in a block digestor (Foss^{TM} Digestor) at 400°C for approximately 70 minutes. During this digestion phase, the organic nitrogen present in the chestnut samples was converted into ammonium sulfate ($(NH_4)_2SO_4$).

After the digestion, the tubes were allowed to cool, and the resulting solution was diluted with 25 mL of distilled water. The subsequent distillation and titration steps were performed automatically by the Kjeldahl equipment. In the distillation phase, the digestate was made alkaline by the addition of sodium hydroxide (NaOH), which converted ammonium sulfate into ammonia (NH_3). The released ammonia was then distilled and captured in a known volume of boric acid solution (H_3BO_3), forming solvated ammonium ions (NH_4^+).

The titration phase consisted of adding standard hydrochloric acid (HCl) to the ammonium-borate complex until a colour change occurred, indicating equivalence point. The volume of acid consumed corresponded to the amount of ammonia produced, and thus to the nitrogen content of the sample.

Protein content was calculated by multiplying the measured nitrogen value by an appropriate conversion factor.

The calculation followed the equation presented below:

$$\textbf{Protein Content}\ (\mathbf{g}) = \textbf{NitrogenContent} \times \textbf{Conversion Factor}\ (\mathbf{5.3}) \quad (5.3)$$

4.2.1.4. Total carbohydrates and Energy

The carbohydrate content was calculated by difference using the following equation:

$$\textbf{Total carbohydrates}(\mathbf{g/100g}) = \mathbf{100} - (\mathbf{g\ ashes} + \mathbf{g\ proteins} + \mathbf{g\ fats})$$

4.2.2. Chemical characterization by different HPLC techniques

4.2.2.1. Free sugars

The identification and quantification of free soluble sugars followed the procedure described by **Barros al. (2013)** with minor modifications. After the Soxhlet extraction, approximately, 1g of the sample (residual solid remaining in the extraction cartridge) was transferred into extraction tubes and mixed with 1mL of melezitose (internal standard,25 mg/mL). Subsequently, 40 mL of an ethanol-water solution (80:20, v/v) were added. The tubes were placed in a water bath at 80 °C for 90 minutes, with manual agitation every 15 minutes to facilitate extraction.

After extraction, the samples were filtered, and the supernatant was transferred into glass flasks. Ethanol was removed by rotatory evaporation at 50 °C under reduced pressure using a Buchi R-210 rotary evaporator (Flawil, Switzerland). The resulting aqueous phase was washed three times with diethyl ether to ensure the efficient removal of non-polar constituents. Each washed phase was transferred to clean tubes and placed in as oven to remove the residual organic solvent, overnight.

The final extracts were brought to a volume of 5 mL with distilled water, filtered, and 1.5 mL of each sample was transferred to HPLC vials for subsequent chromatographic analysis.

The determination of free sugars was performed by High-Performance Liquid Chromatography with refractive index detection (HPLC-RI), using a Knauer Smartline 1000 System equipped with a Knauer Smartline 2300 RI detector (Berlin, Germany). Separation was carried out on a 100-5 NH2 Eurospher column (4.6×250 mm, 5 µm; Knauer). The mobile phase consisted of acetonitrile and deionized water (70:30, v/v), delivered in isocratic mode at the flow rate of 1 mL/min.

Sugar identification was based on the comparison of relative retention times between peaks and authentic standards. Quantification was performed through the internal standard method, using the chromatographic area of melezitose as the internal reference. Results were expressed as g/100g of dry weight.

4.2.2.2. Tocopherols

The extraction of tocopherols was performed using the Soxhlet apparatus **(Figure 9)**, following the same extraction principles previously described for lipid isolation, with the exception of the solvent employed. In this case, hexane was used as the extraction solvent, given its suitability for isolating non-polar compounds such as tocopherols. Approximately 3 g of each sample were placed into cellulose extraction cartridge, and 40 ml of hexane were added to the Soxhlet flasks. After completion of the extraction cycle, the resulting extracts were transferred to pre-weighed tubes and allowed to evaporate under ambient conditions until dryness. Once dry, the tubes were weighed to quantify the recovered extract and subsequently the residue was transferred to HPLC vials for analysis.

Tocopherol analysis was carried out by High-Performance Liquid Chromatography coupled to a fluorescence detector (HPLC-FL). Chromatographic separation was achieved using a normal-phase column (e.g., polyamide or silica-based), with a mobile phase consisting of n-hexane and ethyl acetate (70:30, v/v), operating in isocratic mode. The flow rate was set at 1.0 mL/min, and the injection volume was 20 µL. Detection was performed using a fluorescence detector set at an excitation wavelength of 290 nm and an emission wavelength of 330 nm, conditions commonly used for the selective detection of α-, β-, γ-, and δ-tocopherol isoforms.

Tocopherol identification was achieved through comparison of chromatographic retention times with those of authentic standards. Quantification was performed using calibration curves prepared from commercial tocopherol standards, employing racemic tocol as internal standard. The results were expressed in μg per g of dry weight.

Figure 9 : Soxhlet extractor.

4.2.2.3. Organic acids

Organic acids were analysed following the methodology described by **Roriz et al. (2021)**. Approximately 1 g of each sample was extracted with 25 mL of 4.5% (w/v) metaphosphoric acid solution and magnetically agitated at room temperature for 20 minutes to ensure effective solubilization of the organic acids. The resulting extracts were first filtered through Whatman No.4 filter paper and subsequently passed through a 0.22 μm nylon membrane filter into 1.5 mL amber vials for chromatographic analysis.

Chromatographic separation was performed using a Shimadzu 20A series Ultra-Fast Liquid Chromatography system (UFLC; Shimadzu Corporation, Kyoto, Japan) equipped with a SphereClone reversed phase C18 column (250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA), maintained at 35 °C. The mobile phase consisted of 3.6 mM sulfuric acid, delivered isocratically at a flow rate of 0.8 mL/min. Detection was carried out using a photodiode array detector (PDA), with wavelengths set at 215 nm for general organic acids and 245 nm for ascorbic acid.

Identification and quantification of compounds were accomplished by comparing sample chromatographic retention times and peak areas with calibration curves prepared from commercial standards. Results were expressed in mg per g of dry weight.

4.2.2.4. Fatty acids

The fatty acids composition was determined following the methodology described by to a study described by **Ueda et al. (2021)** with adaptations to ensure consistency with the lipid extraction previously performed. The derivatization of fatty acids into their corresponding fatty acids methyl esters (FAMEs) was carried out by adding 5 mL of methanol/sulfuric acid/toluene solution (2:1:1, v/v/v) to the lipid extract obtained from Soxhlet procedure. The mixture was vortex-mixed and subsequently incubated in a shaking water bath at 50°C (160 rpm) for 12 hours to guarantee complete esterification.

After incubation, 3 mL of distilled water were added to the reaction mixture, followed by vortex agitation for one minute. Then, 3 mL of diethyl ether were added, and the mixture was vortexed again to promote the phase separation. The upper organic layer, containing anhydrous sodium sulfate, ensuring complete dehydration of the extract. The organic phase was them filtered through 0.22 μm membrane filters into 1.5 mL amber vials for gas chromatographic analysis.

Fatty acids profiling was performed using a gas chromatograph (GC) equipped with a split/splitless injector and a flame ionization detector (FID) set to 260 °C. The separation was achieved using a Macherey-Nagel capillary column (50% cyanopropyl-methyl–50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d., 0.25 μm film thickness). The oven temperature program consisted of an initial hold at 50 °C for 2 min; ramp of 30 °C/min to 125 °C; ramp of 5 °C/min to 160 °C; ramp of 20 °C/min to 180 °C; ramp of 3 °C/min to 200 °C; and a final ramp of 20 °C/min to 220 °C, held for 15 min. Hydrogen was used as the carrier gas at a flow rate of 4.0 mL/min (0.61 bar), measured at 50 °C, and injections were performed at 250 °C.

Identification of fatty acids was carried out by comparing the retention times of the sample peaks with those of commercial FAME standards (FAME reference standard mixture, 47885-U, Sigma-Aldrich, St. Louis, MO, USA). Quantification was performed using Claraty software (DataApex, Petrzilkova, Czech Republic), and the results were expressed as relative percentages of each fatty acid.

4.2.2.5. Minerals

The quantification of minerals and metals was performed using Atomic Absorption Spectrometry (AAS) with a Thermo Scientific™ iCE™ 3000 Series spectrometer.

Potassium (K), sodium (Na) and magnesium (Mg) were determined by flame atomization, whereas aluminum (Al), copper (Cu), chromium (Cr), iron (Fe), manganese (Mn), and nickel (Ni) were analysed using electrothermal atomization in a graphite furnace.

For mineral and metal determination, approximately 500 mg of each sample were weighed and subjected to acid digestion using a nitric acid: hydrochloric acid mixture. The digestion was carried out for 20 minutes at 210ºC in a microwave digestion system (Milestone ETHOS X Series). After the digestion process, the vessels were allowed to cool to room temperature. The digested solutions were then filtered and diluted to a final volume of 50 mL with ultrapure water.

Calibration curves were prepared individually for each element, and quantification of minerals and metals was performed in triplicate, using the corresponding calibration curve. All mineral and metal analyses, both identification and quantification, were conducted at the AquaValor Collaborative Laboratory (Chaves, Portugal) by trained technical staff, and not by the student. The results were expressed as g per 100 g of fresh weight.

4.2.3. Bioactive characterization by different bioactive assay

4.2.3.1. Sample preparation

To obtain the hydroethanolic extracts used for the bioactive assays, approximately 1 g of each powdered chestnut sample was mixed with 30 mL of an ethanol–water solution (80:20, v/v). The mixtures were subjected to magnetic stirring for 1 hour at room temperature to ensure efficient extraction of the bioactive constituents. After this period, the extracts were filtered through Whatman No. 4 filter paper, and the solid residues were re-extracted under the same conditions to maximize compound recovery.

The combined filtrates were concentrated by rotary evaporation under reduced pressure using a Büchi R-210 rotary evaporator (Flawil, Switzerland) at 40 °C, until complete removal of the ethanol. The remaining aqueous phase was subsequently freeze-dried to eliminate residual moisture and obtain dry extracts suitable for further bioactive characterization. The lyophilized extracts were stored in sealed containers, protected from light and humidity, until analysis as shown in Figure 10.

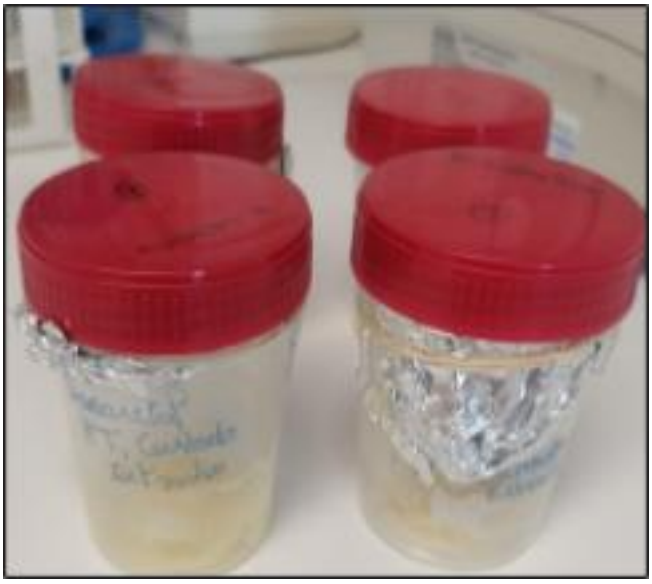


Figure 10: Samples after freeze -drying.

4.2.3.2. Bioactive characterization by antimicrobial assays

4.2.3.2.1 Antibacterial activity

The antibacterial activity of the hydroethanolic extracts was assessed using the microdilution method, following a protocol adapted from **Almeida et al. (2022)** and **Heleno et al. (2013)**. The extracts were tested against eight foodborne bacterial strains of relevance in food safety. The Gram-negative strains included *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginos*a (ATCC 9027), *Salmonella enterica subsp. enterica serovar Typhimurium* (ATCC 13076) and *Yersinia enterocolitica* (ATCC 8610). The Gram-positive strains tested were *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111) and *Staphylococcus aureus* (ATCC 25923). All strains were obtained from Frilabo (Porto, Portugal) and cultured at 37 °C for 24 h in fresh medium to ensure exponential growth before testing.

Bacterial cultures were adjusted spectrophotometrically to 1×10^5 CFU/mL (λ = 625 nm). The extracts were dissolved in a mixture of 5% (v/v) dimethyl sulfoxide (DMSO) and 95% (v/v) tryptic soy broth (TSB) to obtain a stock solution of 20 mg/mL. For the MIC assay, 100 µL of TSB and 100 µL of the extract solution were added to the first row of a 96-well microplate, followed by eight sequential two-fold serial dilutions. Then, 10 µL of the bacterial suspension were added to each well, resulting in a final inoculum of 1×10^5 CFU/mL.

Positive controls consisted of streptomycin, methicillin, and ampicillin, whereas 5% DMSO served as the negative control. Microplates were incubated at 37 °C for 24 h. After incubation, 40 µL of p-iodonitrotetrazolium chloride (INT; 0.2 mg/mL) were added to each well and incubated again for 30 minutes at 37 °C. The minimum inhibitory concentration (MIC) was defined as the lowest extract concentration at which no colour change (absence of INT reduction) was observed, indicating inhibition of visible bacterial growth.

To determine the minimum bactericidal concentration (MBC), all wells showing no colour change during the MIC assay were identified as potential MBCs. The 96-well microplate was incubated again at 37 °C for 24 h, and aliquots from wells without colour development were subcultured onto agar plates. After incubation at 37 °C for 24 h, the lowest concentration showing no colony growth was recorded as the MBC. Results were expressed as mg/mL of the aqueous solutions of the lyophilized extract.

4.2.3.2.2 Antifungal activity

The antifungal activity was evaluated using *Aspergillus fumigatus* (ATCC 1022) and *Aspergillus brasiliensis* (ATCC 16404), both obtained from Frilabo (Porto, Portugal). The strains were maintained on malt agar (MA) at 4 °C and subcultured monthly. Spore suspensions were prepared by washing the surface of MA plates with sterile saline solution (0.85% NaCl) containing 0.1% (v/v) Tween 80. The suspensions were adjusted to 1×10^5 spores/mL, and 100 µL of this inoculum were added per well in 96-well microplates.

MIC determination was performed using serial dilutions of the extracts in malt broth. Microplates were incubated at 28 °C for 72 hours. The MIC was defined as the lowest extract concentration at which no visible fungal growth was observed under optical microscopy relative to the growth control.

The minimum fungicidal concentration (MFC) was determined by transferring 2 µL aliquots from wells without visible growth to new microplates containing 100 µL of malt broth. After incubation at 28 °C for 72 h, the MFC was recorded as the lowest concentration at which no fungal growth was detected, corresponding to 99.5% killing of the initial inoculum. Ketoconazole served as the positive control, while 5% DMSO was used as the negative control.

MIC and MFC values were expressed in mg/mL of the aqueous solutions of the lyophilized hydroethanolic extracts.

4.2.3.2.3 Cytotoxicity

The antiproliferative potential of the chestnut extracts was evaluated using the sulforhodamine B (SRB) colorimetric assay, following previously described procedures with minor adaptations. The biological activity of the extracts was assessed against a panel of cell lines, including human breast carcinoma (MCF-7), human melanoma (MM127), human gastric adenocarcinoma (MKN45), human pancreatic adenocarcinoma (T3M4WC), and the immortalized murine microglial cell line BV-2 **(Figure 11)**. All cell lines were obtained from the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany).

Cancer cell lines were maintained in Roswell Park Memorial Institute (RPMI) medium, while the non-tumour BV-2 cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM). All media were supplemented according to standard cell culture requirements, and cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

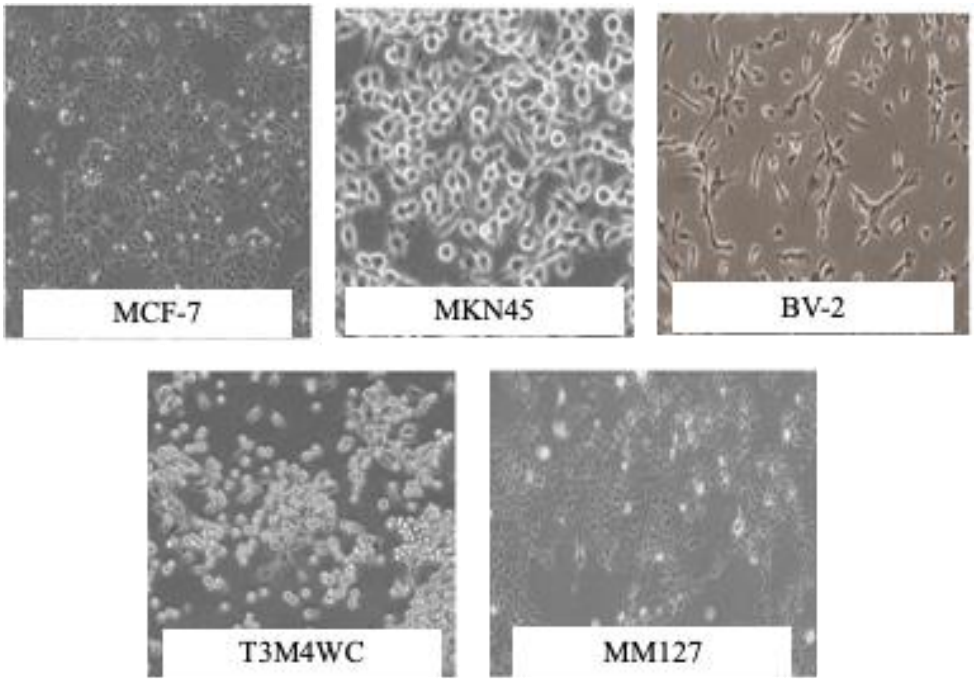


Figure 11: Different cell -Lines used for cytotoxicity assay.

The lyophilized extracts were re-dissolved in distilled water to prepare stock solutions, which were further diluted to obtain a concentration range between 400 and

6.25 μg/mL. Once cell confluence was achieved, the cells were washed with Hank's Balanced Salt Solution (HBSS) and detached using trypsin. After neutralization with culture medium, the cell suspensions were centrifuged at 1200 rpm for 5 minutes. Cell density was adjusted to 1×10^5 cells/mL using a Countess II FL Automated Cell Counter (Invitrogen™, Waltham, MA, USA).

Cells were seeded into 96-well plates and exposed to different concentrations of the extracts for 72 hours under the same incubation conditions. Control wells contained cells cultured in medium without extracts.

For the SRB assay, cell fixation was achieved by adding trichloroacetic acid (TCA; 10%, w/v) to each well, followed by incubation at 4 °C for 1 hour. The plates were then washed thoroughly with water and allowed to dry at room temperature. Subsequently, 100 μL of sulforhodamine B solution was added to each well and incubated for 30 minutes at room temperature. Unbound dye was removed by washing the plates with acetic acid (1%, v/v), after which the plates were dried again. The protein-bound dye was solubilized using Tris buffer (10 mM), and absorbance was measured at 540 nm using a microplate reader.

Cytotoxic activity was expressed as GI_{50} values (μg/mL), defined as the extract concentration required to inhibit 50% of cell growth relative to untreated controls. Ellipticine (Sigma-Aldrich) was used as the positive control.

4.3. Product development

4.3.1. Preparation of the mixture

The development of the chestnut-based mixtures was carried out using four *Castanea sativa* varieties (Amarelal, Boa Ventura, Judia and Longal). For each formulation, equal amounts (50 g) of each chestnut variety were combined, resulting in a homogeneous base mixture. Different volumes of thermal water were then added according to the formulations described in Table 6, in order to obtain a control mixture (without thermal water) and two experimental mixtures containing increasing amounts of thermal water.

After preparation, all mixtures were subjected to freeze-drying for a period of one week to ensure complete moisture removal and product stabilization. The resulting lyophilized samples were subsequently used for further analyses.

Following freeze-drying, the samples were analysed using the same experimental procedures previously described for the raw chestnut samples, employing identical sample amounts, analytical conditions, and methodologies. All nutritional and chemical analyses performed on the individual chestnut varieties were equally applied to the developed mixtures, including the determination of proximate composition according to the official AOAC methods and the characterization of the chemical composition using the chromatographic techniques described in the corresponding methodological sections. This approach ensured methodological consistency throughout the study and enabled a direct comparison between the individual chestnut varieties and the developed formulations regarding their nutritional composition, chemical profile, and bioactive properties.

Table 6: Composition of chestnut mixtures prepared with different amounts of thermal water.

	Control Mixture	***Mixture 1***	***Mixture 2***
Quantity of thermal water (mL)	0	500	250

5. Statistical analysis

All results are expressed as mean ± standard deviation, based on five independent replicates per sample. Data normality was evaluated using the Shapiro–Wilk test, and differences between samples were assessed using one-way analysis of variance (ANOVA).

Whenever the assumption of homogeneity of variances was met, Tukey's test was used for post-hoc comparisons. In cases of heterogeneous variances, Welch's ANOVA followed by the Games–Howell post-hoc test was applied. A significance level of $p < 0.05$ was adopted for all analyses.

6. Results and discussion

6.1. Chestnut varieties

6.1.1. Evaluation of nutritional value of chestnut varieties

The nutritional composition of the four chestnut varieties (Amarelal, Judia, Boa Ventura and Longal) was evaluated through the determination of fat content, ash content, total carbohydrates, crude protein and energy value. In addition, sugars and lipid fractions, particularly saturated fat, were also considered due to their relevance for nutritional quality, sweetness perception and potential technological applications.

Carbohydrates were the predominant nutrient in all samples (**Table 7)**, confirming the typical profile of chestnuts as carbohydrate-rich nuts. The highest carbohydrate contents were recorded in Longal (95.314±0.051g/100g dw) and Amarelal (95.300±0.042 g/100g dw), while Amarelal also presented the highest energy value (399.31±0.22 Kj/100g dw). This result is consistent with major contribution of carbohydrates to the energetic value of chestnuts.

Table 7: Nutritional Value of the chestnut varieties.

Nutritional Value (g/100g dw).				
	Judia	**Longal**	**Amarelal**	**Boa Ventura**
Energy (Kj)	$398.53\pm0.94^{a,b,c}$	397.49 ± 0.31^{c}	399.31 ± 0.22^{a}	398.68 ± 0.32^{b}
Lipids	2.12 ± 0.21^{a}	1.55 ± 0.06^{c}	1.7318 ± 0.0046^{b}	1.82 ± 0.09^{b}
Saturated	$0.503\pm0.02b^{b}$	0.379 ± 0.009^{c}	0.3481 ± 0.0003^{d}	0.63 ± 0.03^{a}
Carbohydrates	94.43 ± 0.22^{c}	95.314 ± 0.051^{a}	95.300 ± 0.042^{a}	95.09 ± 0.09^{b}
Sugars	42.6 ± 0.7^{a}	37.35 ± 0.50^{b}	37.86 ± 0.57^{b}	27.42 ± 0.18^{c}
Proteins	$5{,}66 \pm 0{,}12^{a}$	$5{,}17 \pm 0{,}19^{b}$	$5{,}44 \pm 0{,}15^{a,b}$	$4{,}38 \pm 0{,}21^{c}$
Ash	2.85 ± 0.03^{a}	2.52 ± 0.05^{b}	2.33 ± 0.05^{c}	2.57 ± 0.04^{b}

For each table row, significant differences with $\alpha = 0.05$: $p < 0.001$ between the samples are represented by different letters.

According to **Drăghici et al. (2021) and Li et al. (2022),** the distinctive nutritional value of chestnuts is mainly associated with their high carbohydrate content, predominantly present in the form of starch, rather than with protein and lipid fractions. Compared with other nuts, such as almonds, walnuts and peanuts, chestnuts exhibit the highest starch content. In the present study, the starch content of Judia and

Longal exceeded 50 g/100g on a dry weight basis, suggesting that the varieties may be particularly suitable for flour production. Starch is also relevant for the characteristic sweetness of chestnuts, since during storage it can be partially hydrolysed into glucose, influencing sweetness and post-harvest quality **(Santos et al., 2022).**

Within the carbohydrate fraction, sugars were present in significant amounts and differed among varieties. Judia showed the highest sugar content (42.6±0.7g/100g dw), whereas Boa Ventura presented the lowest (27.42±0.18g/100g dw). These differences may be relevant from both sensory and technological perspectives, since sugar content contributes to sweetness and may influence the suitability of each variety for different food applications. Considering the high carbohydrate levels observed, Longal and Amarelal may also be of interest for applications where sweetness, energy contribution and flour production potential are desirable.

In fact, variations in carbohydrate content are considerably influenced by chestnut origin and genotype **(Musilová et al., 2024)**.

Chestnut varieties were characterized by low lipid content, with the highest value observed in Judia (2.12±0.21 g/100g dw). Saturated fat content was also low in all analysed samples, with Boa Ventura presenting the highest value (0.63g/100g dw) and Amarelal the lowest value (0.3481±0.0003 g/100g dw). These results support the classification of chestnuts as low-fat nuts ,which is consistent with the reported lipid content levels of *C.sativa* (0.70-5.37 g/100g) **(Drăghici et al., 2021)**.

This is also in agreement with previous studies on seventeen native Portuguese chestnuts cultivars, in which fat content ranged from 1.67 to 3.50% of dry matter (**Li et al., 2022).** The low lipid content of chestnuts distinguishes them form other commonly consumed nuts and may contribute to their nutritional interest, particularly in diets aimed at reducing fat intake. Additionally, they represent a good alternative within this food category due to the presence of health-beneficial lipid components **(Drăghici et al., 2021)**.

Protein content was present in appreciable amounts, although at much lower levels than carbohydrates. Judia had the highest concentration (5.66±0.12g/100g dw) while Boa Ventura showed the lowest value (4.38 ±0.21g/100g dw). According to **Li et al. (2022),** proteins represent the second largest component of chestnut dry weight after starch, with total protein contents ranging from 6.0 to 8.6% of dry weight,

depending on the variety. Although the values obtained in the present study are slightly lower than those reported in the literature, they remain comparable and confirm that chestnuts can contribute as a modest source of plant protein.

Regarding ash content, values ranged from 2.33±0.05 g/100g dw in Amarelal to 2.85±0.03g/100g dw in Judia, suggesting a relevant mineral fraction. The higher ash value observed in Judia, together with its higher protein, lipid and sugar contents, indicates that this variety presented the most distinctive nutritional profile among the samples analysed.

Overall, the comparison with previous studies confirms that chestnuts are rich in carbohydrates, particularly starch, and therefore represent an important source of energy. This was especially evident in Amarelal, which showed the highest energy value. The low lipid and saturated fat contents observed across varieties reinforce the nutritional value of chestnuts when compared with other nuts. Additionally, their moderate protein content, relevant sugar levels and mineral fraction support their potential use in food products, particularly flour-based applications. The combination of high carbohydrate content and low lipid levels may also contribute to the beneficial nutritional profile of chestnuts, including potential cardiovascular benefits, as reported by **Santos et al. (2022)**.

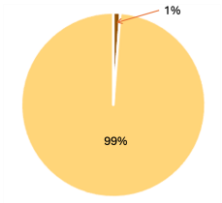
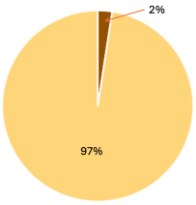
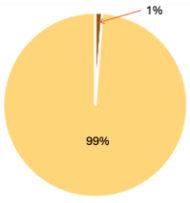
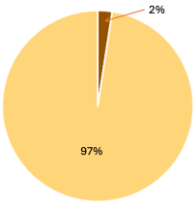
6.1.2. Chemical characterization

6.1.2.1. Free sugars

Following the characterization of total carbohydrates, the profile of soluble free soluble sugars was further analysed by HPLC-RI. This analysis allowed the identification of the main low molecular weight carbohydrate fractions, complementing the overall carbohydrate composition previously discussed.

The results revealed that chestnuts contain mainly sucrose and fructose (**Table 8).** Among these, sucrose was clearly the predominant sugar in all analysed varieties, accounting for approximately 97-99% of total soluble free sugars. This finding is consistent with the high carbohydrate content previously reported and reinforces the role of sucrose as the main contributor to sweetness in chestnuts.

Table 8: HPLC-IR characterization in terms of sugars of the different chestnut varieties.

	Free soluble sugars (g/100g dw).			
	Judia	**Longal**	**Amarelal**	**Boaventura**
Fructose	0.593±0.04^{c}	0.718±0.02^{a}	0.466±0.02^{d}	0.664±0.008^{b}
Sucrose	41.7±1.4^{a}	36.58±0.52^{b}	37.49±0.48^{b}	26.83±0.20^{c}
Total	42.6±0.7^{a}	37.35±0.50^{b}	37.86±0.57^{b}	27.42±0.18^{c}
				

For each table row, significant differences with α = 0.05: p < 0.001 between the samples are represented by different letters.

Significant differences were observed among cultivars. The Judia variety presented the highest sucrose content (41.7±1.4g/100g dw), while Boa Ventura showed the lowest value (26.83±0.20 g/100g dw). These results are in agreement with the variations in total sugars described in the previous section and further support the conclusion that Judia is the variety with the highest sugar content. Such differences may influence both sensory perception and technological performance, particularly in applications where natural sweeteness is desirable.

Fructose was detected in considerably lower amounts compared to sucrose, with the highest value observed in the Longal variety (0.718±0.02g/100g dw). The low levels of fructose highlight the dominance of sucrose within the soluble free sugars fraction and confirm that simple sugars represent only a minor proportion when compared to total carbohydrates, which are mainly present as starch.

A study presented by **Ciucure et al. (2022)** also indicated that sucrose was the dominant sugar identified in Romanian chestnuts .

A comparison among European chestnuts varieties showed that the sucrose content in Romanian, and Turkish chestnuts displayed similar levels (6.820–17.400 g/100g of dw). However, Italian chestnut cultivars presented higher levels of sucrose (0.298–24.509 g/100g of dw) than those cultivated in Spain (3.110–9.940 g/100g dw) **(Antoniewska-Krzeska et al., 2023)**.

The results obtained are in general agreement with those reported **by Barreira et al. (2010)**, who identified sucrose ,fructose and glucose as the main soluble free

sugars in *Castanea sativa.* Mill from Portugal. In that study, sucrose was also the predominant sugar, particularly in Judia (23.30 ± 0.83g/100g dw) and Longal (9.56 ± 0.91g/100g dw) cultivars, while Boa Ventura presented lower values (4.03 ± 0.30g/100g dw).

In contrast, glucose was detected in all varieties analysed by **Barreira et al. (2010)** with the highest value observed in Boa Ventura (6.63 ± 0.49g/100g dw), although always in lower proportions compared to sucrose. For Romanian cultivars, fructose and glucose were quantified at equivalent levels, with fructose ranging between 0.155–1.435 g/100g dw and glucose ranging between 0.156–1.446 g/100g dw **(Ciucure et al., 2022)**.

In the present study, glucose was not detected. This may be explained by its concentration being below the detection limit of the HPLC-RI system used, rather than its complete absence in the samples. Furthermore, differences in soluble free sugars profiles between studies may be attributed to several factors, including geographical origin, environmental conditions, cultivar variability, post-harvest storage, and differences in analytical methodologies.

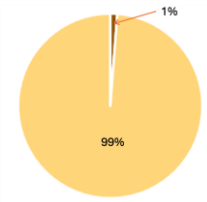
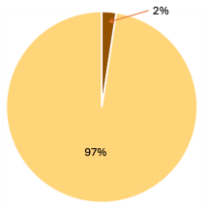
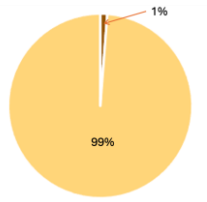
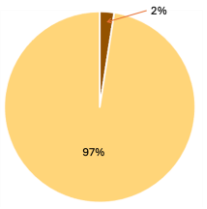
Overall, these results confirm that sucrose is the main soluble free sugar in chestnuts , in agreement with the study performed by **Barreira et al. (2010)** and**, Ciucure et al. (2022)** and reinforce its importance in determining the sweetness and quality attributes of chestnut varieties. In fact, sucrose content is considered one of the significant indicators used for evaluating the quality of chestnuts **(Musilová et al., 2024)**.The predominance of sucrose, together with the previously discussed high starch content, highlights the relevance of carbohydrate composition in defining both the nutritional value and technological potential of chestnuts.

6.1.2.2. Tocopherols

Following the characterization of macronutrients and soluble free sugars, the profile of lipophilic bioactive compounds was further assessed through the determination of tocopherols by HPLC- Fluorescence. Tocopherols, which comprise four isoforms (α-, β-, γ-, and δ-tocopherol), are known for their antioxidant activity and nutritional relevance, particularly in relation to lipid stability and potential health benefits.

In the present study, only two tocopherol isomers, α- and β-tocopherol, were detected in the analysed chestnut varieties (**Table 9**). Among these, β-tocopherol was the predominant form in all samples, with statistically significant differences observed between varieties. The highest content was recorded in the Judia variety (30.58±0.37 µg/100g dw), whereas Boa Ventura exhibited the lowest level (9.052±0.038 µg/ 100g dw). These differences suggest a clear varietal influence on tocopherol composition, which may be relevant for both nutritional quality and oxidative stability.

Table 9*:* HPLC- Fluorescence Characterization in terms of tocopherols of the different chestnut varieties.

	Tocopherols (µg/ 100g dw).			
	Judia	**Longal**	**Amarelal**	**Boaventura**
***α*-tocopherol**	1.03±0.03[a]	0.326±0.006[c]	0.84±0.02[b]	0.249±0.007[d]
***β*-tocopherol**	30.58±0.37[a]	24.873±0.017[c]	29.154±0.021[b]	9.052±0.038[d]
Total	31.74±0.41[a]	25.192±0.023[c]	30.001±0.037[b]	9.321±0.054[d]
				

For each table row, significant differences with α = 0.05: p < 0.001 between the samples are represented by different letters.

α -tocopherol, the second isoform identified, was present at considerably lower concentrations across all varieties. The highest value was observed in Judia (1.03±0.03µg/100g dw), while Boa Ventura again showed the lowest content (0.249±0.007µg/100g dw). Despite its lower concentration, α-tocopherol is often considered the most biologically active form of vitamin E, which highlights the importance of its presence even at reduced levels.

Results reported for Turkish chestnuts cultivars are in good agreement with the lower concentrations of α -tocopherol observed in the present study, with values around 1.9µg/100g dw; however, **Kiralan et al. (2019)** identified γ-tocopherol, as the predominant isomer reaching levels of approximately 1000µg/100g dw ,whereas β-tocopherol not detected in those cultivars.

The predominance of β-tocopherol over α -tocopherol observed in the present study is consistent with the overall low lipid content in chestnuts, previously discussed, since tocopherols are lipid soluble compounds, and their concentration is generally associated with the lipid fraction. The variation observed among varieties in the may therefore be related not only to genetic factors but also to differences in lipid composition and distribution.

According to **Li et al. (2022),** chestnuts present higher levels of tocopherols compared to other nuts ,particularly almonds, reinforcing their potential as a source of antioxidant compounds. Although the total tocopherol content detected in the present study is relatively low in absolute terms, its presence contributes to the nutritional value of chestnuts, particularly in terms of antioxidant capacity and potential health promoting effects.

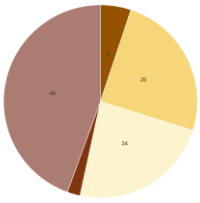
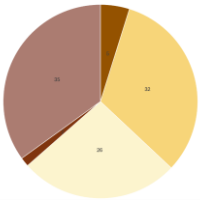
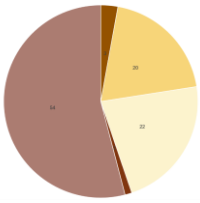
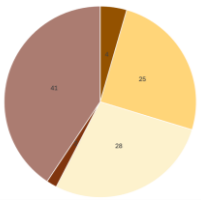
Overall, the results indicate that tocopherol composition varies among chestnut varieties, with Judia consistently presenting higher levels of both β- and α-tocopherol. These findings complement the previously discussed nutritional profile and highlight the contribution of minor bioactive compounds to the overall quality and functional value of chestnuts.

6.1.2.3. Organic acids

Following the characterization of macronutrients, soluble sugars and tocopherols, the profile of organic acids was further evaluated by UFLC-PDA. Organic acids are important constituents that contribute to the sensory properties, stability and nutritional value of chestnuts.

The results **(Table 10)** showed that five organic acids were identified in the analysed chestnut varieties: oxalic, quinic, malic, ascorbic, and citric acids, with statistically significant differences observed among the samples. Among these compounds, citric acid was the predominant organic acid in all varieties, reaching a maximum concentration of 3.87±0.15 g/100g dw in the Amarelal variety. This highlights its major contribution to the organic acid profile of chestnuts and its potential influence on taste and preservation. In addition, citric acid is well known for its metabolic importance **(Şengül et al., 2023).**

Table 10: UFLC-PDA Characterization in terms of organic acids of the different chestnut varieties.

	Organic Acids (g/100g dw).			
	Judia	**Longal**	**Amarelal**	**Boaventura**
Oxalic Acid	0.194±0.015^{b}	0.249±0.009^{a}	0.200±0.01^{b}	0.168±0.003^{c}
Quinic Acid	0.93±0.02^{c}	1.69±0.09^{a}	1.41±0.03^{b}	0.953±0.028^{c}
Malic Acid	0.896±0.028^{d}	1.37±0.05^{b}	1.60±0.07^{a}	1.04±0.03^{c}
Ascorbic Acid	0.0816±0.0009^{a}	0.0785±0.0009^{b}	0.0820±0.0016^{a}	0.0661±0.0004^{c}
Citic Acid	1.69±0.04^{c}	1.80±0.02^{b}	3.87±0.15^{a}	1.53±0.02^{d}
				

For each table row, significant differences with α = 0.05: p < 0.001 between the samples are represented by different letters.

Furthermore, quinic acid, a precursor used in the synthesis of various pharmaceuticals (Ciucure et al., 2022), was identified in the present study, with the highest concentration of 1.69±0.09 g/100g showing with Longal.

In contrast, ascorbic acid was found at the lowest concentrations among the identified organic acids. Values ranged from 0.0661±0.0004 g/100g dw in Boa Ventura to 0.0820±0.0009 g/100g dw in Amarelal, with Judia also presenting relatively high levels (0.0816±0.0009 g/100g dw). Despite being present in lower amounts, ascorbic acid is nutritionally relevant due to its well-known antioxidant properties and contribution to vitamin C intake.

The results obtained in this study are partially consistent with those presented by **Delgado et al. (2018)**, who analysed two Portuguese chestnut varieties (Judia and Longal). In that study, citric acid was also identified as the main organic acid; however, lower concentrations were reported, with a maximum value of 0.955±1,2 g/100g dry matter in Judia. In contrast, ascorbic acid showed higher values compared to the present study, reaching 0.108±0.4 g/100g dry matter for Judia and 0.080±03 g/100g dry matter for Longal.

In comparison with chestnuts from Spain, the average citric acid concentrations were found to range between 0.0261 and 0.1135 g/100g, while the highest concentration for the ascorbic acid surpassed 0.08g/100g **(Suárez et al., 2012)**.

Additionally, **Delgado et al., (2018)** reported the presence of fumaric acid, which was not detected in the present study, whereas oxalic and quinic acids identified in this work were not reported in their results. Both acids were previously found in a Portuguese chestnuts study with concentrations of 0.07 g/100g dw for oxalic acid and 1.3 g/100g dw for quinic acid **Carocho et al.(2013)**. These concentrations were considered low comparing to the present results.

These discrepancies in the qualitative and quantitative profiles of organic acids may be attributed to several factors, including differences in cultivar, geographical origin ,soil conditions, state of maturity, cultural practices, storage conditions, and the time elapsed between harvest and analysis, as highlighted by **(Delgado et al., 2018; Şengül et al., 2023).** Methodological differences between analytical techniques may also contribute to these variations.

Overall, the present results indicate that citric acid is the dominant organic acid in chestnuts, while ascorbic acid, although present at lower levels, contributes to their nutritional value. The variability observed among varieties reinforces the influence of genetic and environmental factors on organic acid composition and highlights their role in the sensory and functional properties of chestnuts.

6.1.2.4. Fatty acids

Following the characterization of the other nutritional components, the fatty acid profile of the analysed chestnuts varieties was determined by GC-FID, and the results are expressed as relative percentages (**Table 11**). The fatty acids composition provides important information regarding the nutritional quality and potential health implications of chestnut consumption.

Overall, unsaturated fatty acids were the predominant fraction in most of the analysed varieties, particularly Judia, Longal and Amarelal. In these samples, Polyunsaturated Fatty Acids (PUFAs) represented the main group, with values ranging from 44.00±0.06 in Longal to 52.36±0.04% in Amarelal. This high proportion of PUFAs is mainly attributed to linoleic acid (C18:2n6c), which was identified as the most abundant fatty acid in these varieties, reaching 43.66±0.03% in Amarelal. In addition, α-linolenic acid (C18:3n3), an essential omega-3 fatty acid, was also present in relevant amounts, particularly in Amarelal (8.685±0.009%) and Judia (7.71±0.03%). These findings highlight the nutritional importance of these varieties,

as both linoleic and α-linolenic acids are essential fatty acids associated with beneficial physiological functions.

In contrast, the Boa Ventura variety exhibited a markedly different fatty acid profile, with the highest level of Monounsaturated fatty acids (MUFAs) as the dominant group (48.76±0.16%). This was largely due to its high oleic acid content (C18:1n9c), which reached 46.94±0.18%, substantially higher than in the remaining varieties. Oleic acid is widely recognized for its beneficial effects on cardiovascular health, which may confer additional nutritional value to this specific variety.

Saturated fatty acids (SFAs) represented the third major group in all samples, with the highest proportion again observed in Boa Ventura variety (35.70±0.25%), compared to lower values in Amarelal (20.092 ± 0.005%), Judia (23.00 ± 0.28%) and Longal (25.51 ± 0.44%). Among SFAs, palmitic acid (C16:0) was the most prevalent in all varieties, with values ranging from 17.124±0.002 in Amarelal to 28.08±0.24 in Boa Ventura. Stearic acid (C18:0) was also present, although at lower concentrations. Minor saturated fatty acids such as C14:0, C15:0 and long-chain SFAs (C20:0–C24:0) were detected in small amounts, contributing to the overall fatty acid profile.

The results obtained are in agreement with previous studies on Portuguese chestnuts. According to **Massantini et al. (2021),** chestnuts are characterized by a high proportion of unsaturated fatty acids, accounting for approximately 83% of total fatty acids .

Findings from **(Barreira et al., 2009)** on four Portuguese chestnuts showed that three major fatty acids were identified: linoleic(C18:2n6c), oleic(C18:1n9c) and palmitic (C16:0) acids. PUFAs were the most abundant fraction with high proportions of linoleic acid. The MUFA content, in this study, varied from 30.9% to 38.7%. Palmitic acid was also identified as the principal saturated fatty acid with values ranging from 14.3% to 17.3%, which is consistent with the present findings.

Furthermore, saturated fatty acids were reported at relatively low levels (around 17%) **(Massantini et al., 2021)**, which, although slightly lower than those observed in this study, particularly in Boa Ventura, still supports the classification of chestnuts as a source of predominantly unsaturated fats.

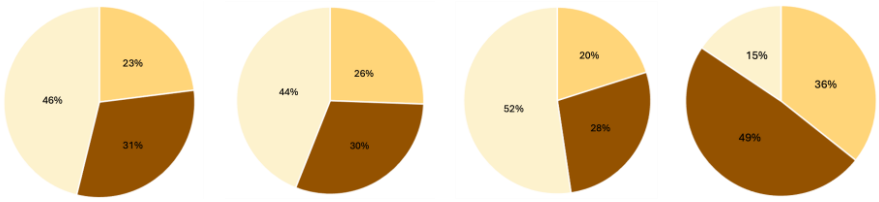
The variability observed among varieties may be attributed to genetic factors, environmental conditions, and cultivation practices, which are known to influence

lipid composition. Notably, the distinct profile of Boa Ventura, with higher MUFA and SFA contents and lower PUFA levels, contrasts with the other varieties, which are more enriched in polyunsaturated fatty acids.

Overall, chestnuts could be considered a valuable source of essential fatty acids and their fatty acid composition confirm their nutritional relevance, particularly due to the predominance of unsaturated fatty acids. The high levels of linoleic acid, combined with the presence of α-linolenic acid, support the potential health benefits associated with chestnut consumption, especially in relation to cardiovascular health ,and metabolic function, insulin action, neuronal development, and visual function as highlighted by **Barreira et al. (2009), Massantini et al. (2021), and Antoniewska-Krzeska et al. (2023).**

Table 11: CG-FID Lipidic fraction and fatty acids composition of the chestnut varieties.

	Fatty acids (%)			
Compound Name	**Judia**	**Longal**	**Amarelal**	**Boaventura**
C6:0	0.305±0.002^{c}	0.640±0.007^{b}	n.d.	0.749±0.007^{a}
C8:0	0.059±0.001^{b}	n.d.	n.d.	0.197±0.002^{a}
C10:0	0.026±0.008	n.d.	n.d.	n.d.
C12:0	0.051±0.001^{c}	0.123±0.003^{a}	n.d.	0.1094±0.0005^{b}
C14:0	0.191±0.005^{c}	0.312±0.004^{b}	0.195±0.001^{c}	0.319±0.003^{a}
C15:0	0.230±0.001^{c}	0.359±0.010^{b}	0.174±0.003^{d}	0.573±0.017^{a}
C16:0	18.94±0.25^{c}	20.34±0.40^{b}	17.124±0.002^{d}	28.08±0.24^{a}
C16:1	0.651±0.005^{b}	0.657±0.010^{b}	0.576±0.004^{c}	0.913±0.016^{a}
C17:0	0.180±0.001^{d}	0.2664±0.0005^{b}	0.221±0.007^{c}	0.352±0.010^{a}
C18:0	1.525±0.008^{c}	1.96±0.03^{b}	1.218±0.011^{d}	2.555±0.006^{a}
C18:1n9c	29.52±0.12^{b}	29.14±0.31^{b}	26.44±0.02^{c}	46.94±0.18a
C18:2n6c	38.40±0.14^{b}	37.17±0.12^{c}	43.66±0.03^{a}	13.71±0.03^{d}
C18:3n3	7.71±0.03^{b}	6.66±0.06^{c}	8.685±0.009^{a}	1.510±0.003^{d}
C20:0	0.536±0.003^{b}	0.5692±0.0004^{a}	0.336±0.002^{c}	1.09±0.01^{d}
C20:1	0.640±0.004^{b}	0.634±0.002^{b}	0.544±0.007^{c}	0.886±0.011^{a}
C22:0	0.557±0.007^{c}	0.680±0.007^{b}	0.538±0.010^{d}	0.92±0.04^{a}
C22:2	0.157±0.001^{c}	0.236±0.006^{b}	n.d.	0.363±0.005^{a}
C23:0	0.145±0.003^{b}	n.d.	n.d.	0.248±0.007^{a}
C24:0	0.333±0.004^{c}	0.417±0.002^{b}	0.292±0.007^{d}	0.525±0.008^{a}
SFA	23.00±0.28^{c}	25.51±0.44^{b}	20.092±0.005^{d}	35.70±0.25^{a}
MUFA	30.78±0.12^{b}	30.45±0.30^{b}	27.57±0.04^{c}	48.76±0.16^{a}
PUFA	46.17±0.16^{b}	44.00±0.06^{c}	52.36±0.04^{a}	15.59±0.03^{b}



Relative to fatty acid identification: Palmitic acid (C16:0); stearic acid (C18:0); oleic acid (C18: 1n9c); linoleic acid (C18:2n6c); α-linolenic acid (C18:3n3); Saturated fatty acids (SFA); Monounsaturated fatty acids (MUFA); Polyunsaturated fatty acids (PUFA). For each table row, significant differences with α = 0.05: p < 0.001 between the samples are represented by different letters. nd - not detected.

6.1.2.5. Minerals

Following the characterization of the chemical composition of chestnut varieties, the mineral profile was determined by AAS and expressed as relative percentages to facilitate comparison among samples **(Figure 12)**. Seven minerals were identified, namely potassium (K), magnesium (Mg), iron (Fe), copper (Cu), manganese (Mn), aluminum (Al) and nickel (Ni), revealing differences in their relative distribution across the analysed varieties.

The graphical representation clearly shows that potassium is the predominant mineral in all chestnut varieties, accounting for the vast majority of the total mineral composition. This marked predominance highlights the nutritional relevance of potassium in chestnuts and confirms its role as the main mineral constituent. Among the analysed varieties, Boa Ventura appears to present the highest relative proportion of potassium, although all samples exhibit a very similar dominance of this element.

Magnesium is the second most abundant mineral across all varieties, although its relative contribution is considerably lower than that of potassium. Even so, it represents the most relevant mineral after potassium and contributes meaningfully to the overall mineral profile.

The remaining minerals (Fe, Cu, Mn, Al and Ni) are present in much smaller proportions, as evidenced by their minimal representation in the graphical profile. Among these trace elements, iron and manganese appear to contribute more noticeably than copper, aluminum and nickel, although all remain minor components of the overall mineral composition.

Nickel is present in all varieties at very low relative levels, showing minimal variation between samples. Similarly, aluminum and copper contribute only marginally to the total mineral profile, although slight varietal differences can still be observed. In particular, Boa Ventura appears to show a relatively higher contribution of copper, while Longal presents a comparatively higher proportion of aluminum.

Different studies on sweet chestnuts from other European countries, such as Spain and Slovakia, revealed that chestnuts are a rich source of microelements, especially Potassium, which was identified as the most abundant mineral among different cultivars. Calcium and sodium were both detected, despite the variability in

their concentrations observed ,which contrasts with the present findings**(Ciucure et al., 2022; Musilová et al., 2024)**.

From a varietal perspective, the graphical analysis indicates that all samples are characterized by a higher relative contribution of several minerals, particularly potassium and magnesium. These differences do not allow us to conclude whether mineral composition is influenced by varietal characteristics, even when expressed on a relative basis. The predominance of potassium and the relevance of magnesium observed in this study are consistent with previous findings, which identify chestnuts as an important source of essential minerals. Potassium is considered as an important mineral for the human body, while Magnesium is essential for many physiological processes such as neuromuscular function, cellular energy metabolism, and receptor activation**(Ciucure et al., 2022; Razzaque & Wimalawansa, 2025).**

Variations observed among varieties may be attributed to factors such as soil composition, environmental conditions and cultivation practices, which are known to influence mineral uptake in plants (**Santos et al., 2022).**

Overall, the mineral composition of the analysed chestnut varieties is characterized by a clear dominance of potassium, followed by magnesium, while the remaining elements are present in trace proportions. The use of relative percentages allows for a clearer comparison between varieties, highlighting subtle differences in mineral distribution and reinforcing the nutritional importance of chestnuts as a source of essential minerals that support human health through enzyme activation and several functions**(Musilová et al. , 2024).**

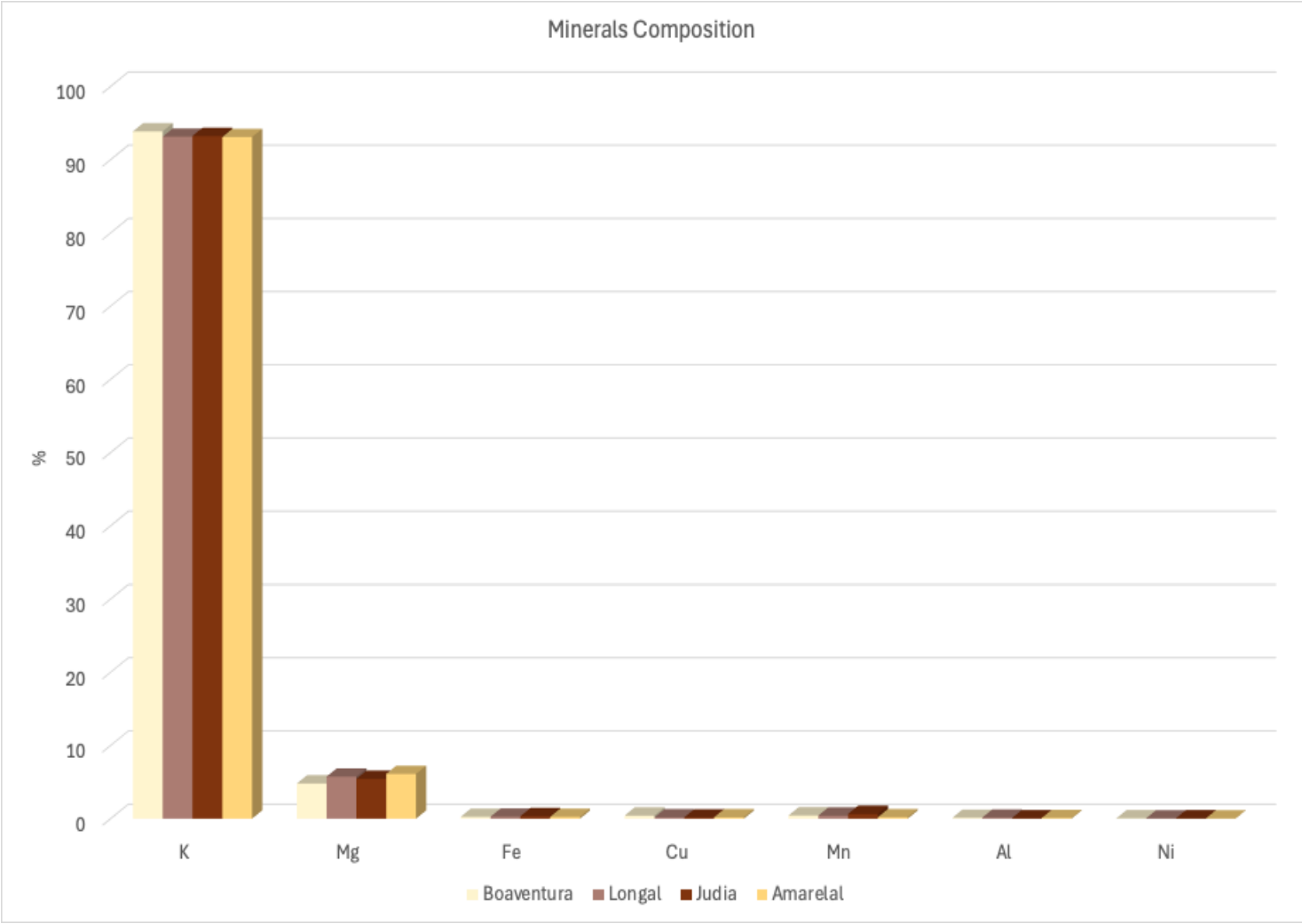


Figure 12: Mineral contents of the different varieties of chestnut expressed in percentages.

6.1.3. Bioactive characterization of chestnuts varieties

6.1.3.1. Antibacterial activity and Antifungal activity

Following the chemical characterization of the analysed chestnuts varieties, their antimicrobial potential, including both antibacterial and antifungal activities, was evaluated. The antimicrobial activity results are presented in Table 12.

Regarding the antibacterial activity, the extracts from all chestnut varieties were tested against a panel of Gram-positive bacteria, including *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus*, as well as Gram negative strains such as *Enterobacter cloacae*, *Escherichia coli*, *Salmonella enterica* and *Yersinia enterocolitica*. Overall, the results indicated limited antibacterial activity, as both MIC and MBC values were higher than 10 mg/mL for all tested microorganisms. This suggests that, under the experimental conditions applied, the chestnut extracts did not exhibit significant antimicrobial effects. The low activity observed may be related to the concentration range tested, which may not have been sufficient to induce inhibition, indicating that higher concentrations (>10 mg/mL) could potentially enhance the antibacterial effect.

Similarly, the antifungal activity of chestnut extracts was evaluated against *Aspergillus brasiliensis* and *Aspergillus fumigatus*. All samples exhibited limited antifungal activity, with both MIC and MFC values exceeding 10 mg/mL. These results indicate that relatively high concentrations of chestnut extracts are required to inhibit fungal growth, suggesting a weak antifungal potential under the tested conditions, once more.

The limited antimicrobial activity observed in both assays may be associated with the intrinsic chemical composition of chestnuts. In particular, their high carbohydrate and sugar content may favour microbial growth rather than inhibition, especially in the case of fungi. Additionally, post-harvest conditions may further facilitate fungal contamination and development, as reported by **Li et al. (2022),** who highlighted the impact of fungal communities on chestnut decay.

In contrast, studies focusing on chestnut byproducts have reported more pronounced antimicrobial effects. **Silva et al. (2020)** investigated the antimicrobial activity of phenolic extracts obtained from chestnut industry by-products, including the inner and outer shells, burs, and leaves, particularly from the Longal variety. Their

results demonstrated that these extracts exhibited significant antimicrobial activity, especially against Gram-positive bacteria such as *Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus,* and *Staphylococcus epidermidis,* with the inner shell extract showing the highest effectiveness.

These differences suggest that the antimicrobial potential of chestnuts derived materials is strongly influenced by their phenolic composition. While the edible portion of the fruit appears to have limited antimicrobial activity, likely due to lower concentrations of bioactive compounds, byproducts may represent a richer source of phenolic compounds with known antimicrobial properties. Furthermore, factors such as extraction method, solvent type and compound stability may also contribute to the variability observed between studies.

Overall, the results indicate that chestnut fruits exhibit limited antibacterial and antifungal activity under the tested conditions. However, the comparison with literature highlights the importance of phenolic compounds in antimicrobial activity and suggests that chestnut byproducts may represent a more promising source of bioactive compounds with potential applications in food preservation and health related fields (**Silva et al., 2020; Li et al. 2022).**

Table 12*:* Antibacterial and antifungal activity of chestnut varieties.

Antibacterial activity														
									Positive control					
	Judia		**Longal**		**Amarelal**		**Boa Ventura**		**Streptomicin**		**Methicilin**		**Ampicillin**	
	10mg/mL		**10mg/mL**		**10mg/mL**		**10mg/mL**		**1mg/mL**		**1mg/mL**		**20mg/mL**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria														
Enterobacter cloacae	>10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t	0.15	0.15
Escherichia coli	>10	>10	>10	>10	>10	>10	>10	>10	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterica	>10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	>10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria														
Bacillus cereus	>10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	>10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	>10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	0.007	0.007	0.15	0.15

Antifungal activity										
									Positive control	
	Judia		**Longal**		**Amarelal**		**Boa Ventura**		**Ketoconazole**	
	10mg/mL		**10mg/mL**		**10mg/mL**		**10mg/mL**		**1mg/mL**	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MBC**	**MIC**	**MFC**
Fungi										
Aspergillus brasiliensis	>10	>10	>10	>10	>10	>10	>10	>10	0.06	0.125
Aspergillus fumigatus	>10	>10	>10	>10	>10	>10	>10	>10	0.5	1

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MFC: minimal fungical concentration nt: not tested.

6.1.3.2. Cytotoxicity

Following the evaluation of antimicrobial activity, the cytotoxic potential of chestnut extracts was assessed by determining their ability to inhibit 50% of cell proliferation (GI_{50}) as detailed in Table 13. This parameter provides an indication of both potential antitumor activity and safety profile.

Table 13*:* Cytotoxicity of chestnut varieties.

	Cytotoxicity (GI50, µg/mL)			
	Judia	**Longal**	**Amarelal**	**Boa Ventura**
MCF7	>400	>400	>400	>400
MM127	>400	>400	>400	>400
T3M4WC	>400	>400	>400	>400
MKN45	>400	>400	>400	>400
BV2	>400	>400	>400	>400

The results showed that all the tested extracts obtained from the four varieties of chestnuts exhibited GI_{50} values exceeding 400 µg/mL for all tested cell lines including tumour cell lines (MCF-7; MM127; MKN45; T3M4WC) as well as non-tumour cells (BV-2). These values indicate that, under the experimental conditions applied, the extracts did not significantly inhibit cell proliferation. Consequently, the analysed chestnut extracts can be considered as non-toxic toward both cancerous and normal cells within the tested concentration range.

The absence of toxic effects suggests a favourable safety profile for chestnut extracts, which may be advantageous for their potential use in food or nutraceutical applications. However, this also reflects a limited antitumour potential under the conditions tested, as no relevant antiproliferative activity was observed.

In contrast, previous studies have reported different outcomes depending on the plant material analysed. **Gomes-Laranjo et al. (2025)** reviewed the cytoxic effects of various parts of the European chestnut, including leaves, burs, shells and inner shells, and highlighted that these extracts may exhibit antitumoral activity against different cell lines. These findings suggest that nonedible parts of the chestnut may contain higher concentrations of bioactive compounds capable of inducing cytotoxic effects.

The discrepancies between the present results and those reported in the literature may be explained by several factors, including the concentration of extracts tested, the specific plant part used, and the type of cell line evaluated. In particular, the edible portion of chestnuts may contain lower levels of bioactive compounds associated with antiproliferative activity when compared to byproducts such as shells or leaves. Additionally, methodological differences in extraction procedures and experimental design may also contribute to the observed variability.

Overall, the results indicate that chestnut fruit extracts do not exhibit significant cytotoxic effects under the tested conditions, supporting their safety for consumption. However, the comparison with previous studies suggests that other parts of the chestnut plant may represent a more promising source of compounds with potential antitumour activity **(Gomes-Laranjo et al., 2025)**.

6.2. Chestnut Mixtures

6.2.1. Evaluation of nutritional value of chestnut mixtures

The nutritional composition of the three chestnut mixtures (control mixture, mixture 1 containing 500 ml of thermal water and mixture 2 containing 250 ml of thermal water) was assessed, as previously performed for the chestnut varieties, through the determination of fat content, ash content, total carbohydrates, crude protein and energy content, as well as free sugars, and lipid profiles, specifically saturated fat.

The nutritional analysis detected notable differences among the chestnut mixtures, particularly in those enhanced by thermal water, highlighting variability in fat, ash, free sugars, and lipids contents **(Table 14)**.

Carbohydrates were again the most abundant nutrient in all mixtures, with relatively close levels among samples, resulting in high energy values.

Free sugars also were present in considerable values. The control mixture showed a slightly higher sugar content (46.6±0.5g/100g dw) comparing to mixture 1 (43.33±0.32 g/100g dw) and mixture 2 (45.0±1.0 g/100g dw), with mixture 2 presenting intermediate values. The lower sugar content observed in the thermal water mixtures may be related to the dilution effect caused by water addition.

Table 14: Nutritional Value of the different mixtures.

Nutritional Value (g/100g dw).			
	Control Mixture	**Mixture 1**	**Mixture 2**
Energy (Kj)	401.00±0.19[a]	395.58±0.23[c]	396.6±0.7[b]
Lipids	1.69±0.04[a]	1.21±0.06[b]	1.149±0.015[b]
Saturated	0.288±0.005[b]	0.75±0.03[a]	0.756±0.012[a]
Carbohydrates	95.81±0.16[a]	95.45±0.12[b]	95.94±0.13[a]
Sugars	46.6±0.5[a]	43.33±0.32[c]	45.0±1.0[b]
Proteins	5.62 ± 0,28	5,70 ± 0,54	5,72 ± 0,37
Ash	1.87±0.09[c]	2.66±0.10[a]	2.29±0.12[b]

For each table row, significant differences with $\alpha = 0.05$: $p < 0.001$ between the samples are represented by different letters.

In light of the high carbohydrate concentrations detected, the addition of thermal water did not appear to significantly affect the carbohydrate profile, therefore preserving the energy potential of the mixtures.

All the three mixtures were characterized by low fat contents, ranging between 1.69±0.04g/100gdw and 1.149±0.015g/100g dw. Saturated fats were also present in all mixtures, with slightly higher levels observed in the mixtures containing thermal water compared to the control.

The low lipid content enhances the nutritional suitability of these mixtures for consumer consumption, as low-fat foods helps in reducing blood cholesterol levels **(Musilová et al., 2024)**.

Protein content was present in acceptable amounts and remained relatively similar among the three mixtures, with values of 5,70±0,54 g/100g dw for mixture 1 and 5,72±0,37g/100g dw for mixture2.These findings are in agreement with the assumption that the addition of thermal water did not negatively affect protein preservation.

Concerning ash content, the mixtures with thermal water presented higher values, with 2.66±0.10g/100g dw for mixture 1 and 2.29±0.12 g/100g dw for mixture 2 compared to the control mixture 1.87±0.09 g/100g dw. This increase highlights the positive contribution of thermal water to the mineral composition of the products, as thermal water is known from literature for its high mineral content.

Furthermore, these results suggested that increasing the volume of thermal water corresponded to an increase in ash content.

Overall, the comparison between chestnuts mixtures revealed the beneficial effect of thermal water in enhancing the nutritional profile of the final product, thereby potentially contributing to health benefits for consumers.

6.2.2. Chemical characterization

6.2.2.1. Free sugars

Subsequent to the nutritional value evaluation, the chemical composition of chestnut mixtures was further characterized through various analyses, beginning with the free sugar profile, which was analysed by HPLC-RI, as previously mentioned. This analysis allowed the characterization of the soluble free sugar fraction present in the mixtures.

The results of soluble free sugars revealed (**Table15**) that the three mixtures contain only sucrose. The sucrose concentration of the control mixture (46.6±0.5g /100g dw) was higher than those observed in mixture 1 and mixture 2. Moreover, mixture 2 exhibited a slightly higher sucrose value (45.0±1.0g/100g dw) compared to mixture 1 (43.33±0.32g/100g dw). Such variability may be explained by the dilution effect associated with the addition of thermal water.

Table 15*:* HPLC-IR Characterization in terms of sugars of the different mixtures.

Free sugars (g/ 100g dw).

	Control Mixture	**Mixture 1**	**Mixture 2**
Sucrose	46.6±0.5^{a}	43.33±0.32^{c}	45.0±1.0^{b}
Total	46.6±0.5^{a}	43.33±0.32^{c}	45.0±1.0^{b}

For each table row, significant differences with α = 0.05: p < 0.001 between the samples are represented by different letters.

Overall, these findings showed that the addition of thermal water did not significantly affect the sucrose content of the final products, despite its slight reduction observed in the enhanced mixtures. In al samples, sucrose remained the predominant free sugar, preserving the characteristic sweetness of chestnut based products.

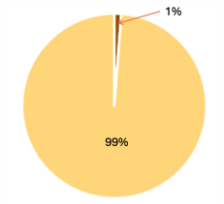
Sucrose proved to play an important role in contributing to the sweetness of the final product and widely known as one of the abundant sweeteners, playing an essential role in both human diet and the food industry **(Ni et al., 2022).**

6.2.2.2. Tocopherols

The second analysis of chemical composition of the chestnut mixtures involved the identification of tocopherols by HPLC-Fluorescence. Tocopherols are important lipophilic bioactive compounds associated with antioxidant activity and oxidative stability.

The results (**Table 16**) showed that β-tocopherol was present in all the three mixtures, with statistically significant differences among them.

Table 16 :HPLC- Fluorescence Characterization in terms of tocopherols of the different mixtures.

Tocopherols (µg /100g dw).			
	Control Mixture	**Mixture 1**	**Mixture 2**
***α*-tocopherol**	0.22±0.04	n.d.	n.d.
***β*-tocopherol**	49.23±0.16[a]	12.55±0.32[b]	4.29±0.17[c]
Total	49.45±0.17[a]	12.55±0.32[b]	4.29±0.17[c]
			

For each table row, significant differences with α = 0.05: p < 0.001 between the samples are represented by different letters. nd: not detected.

The highest concentration of β-tocopherol was detected in the control mixture (49.23±0.16 µg g/100g dw), whereas the enriched mixtures showed lower levels. Among the enhanced, mixture 1 contained a higher content (12.55±0.32 µg /100g dw) than mixture 2 (4.29±0.17µg /100g dw). This reduction observed in β-tocopherol in the two mixtures may be associated with their lower lipid content, since tocopherols are lipid-soluble compounds.

However, α -tocopherol, was found only in the control mixture, at low levels (0.22±0.04µg /100g dw) and was not identified in the other two mixtures.

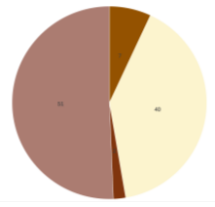
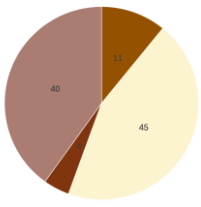
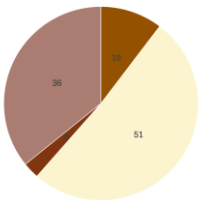
Overall, the differences detected in this study in tocopherol isomer concentrations suggest that the addition of thermal water may affect the stability of tocopherols, especially α -tocopherol. Nevertheless, β -tocopherol appeared to be more stable under these conditions, remaining detectable in all analysed mixtures.

6.2.2.3. Organic acids

Following the characterization of soluble sugars and tocopherols, the organic acid profile of was further analysed by UFLC-PDA.

Essential organic acids for human health, such as citric, malic, oxalic, and ascorbic acids were identified **(Table 17)**. Citric acid was the predominant acid among all the three mixtures, with the highest concentration presented by the control mixture (1.853±0.008 g/100g dw). Lower levels were detected in the enriched mixtures, with 1.00±0.02g/100g dw for mixture 2 and 0.63±0.02g/100g dw for mixture1. Malic acid, identified as the second most abundant organic acid, remained at relatively stable concentrations between the control mixture (1.47±0.08g/100g dw) and mixture 2 (1.42±0.06 g/100g dw).

Table 17*:* UFLC-PDA Characterization in terms of organic acids of the different chestnut mixtures.

	Organic Acids (g/ 100g dw).		
	Control Mixture	**Mixture 1**	**Mixture 2**
Oxalic Acid	0.2530±0.0026^{b}	0.170±0.005^{c}	0.286±0.012^{a}
Quinic Acid	n.d.	n.d.	n.d.
Malic Acid	1.47±0.08^{a}	0.70±0.04^{b}	1.42±0.06^{a}
Ascorbic Acid	0.0752±0.0015^{a}	0.0680±0.0004^{b}	0.0758±0.0020^{a}
Citic Acid	1.853±0.008^{a}	0.63±0.02^{c}	1.00±0.02^{b}
			

For each table row, significant differences with α = 0.05: p < 0.001 between the samples are represented by different letters. nd: not detected.

However Ascorbic acid was found at low concentrations in all three mixtures. Its values ranged from 0.0758±0.0020 g/100g and 0.0680±0.0004 g/100g dw with similar levels again observed between the control and mixture 1.

The overall reduction in organic acid overall concentration observed in the enriched mixtures could be caused by the addition of thermal water which may have led to a dilution effect.

Overall, the present results highlight that citric acid is the major organic acid in all analysed mixtures, while ascorbic acid was present in lower amounts. The combination of these compounds, together with the other organic acids detected, contributes to the nutritional and functional potential of the final product.

Citric acid plays a crucial role in metabolism by providing energy to the body. In addition, despite its low levels, oxalic acid can enhance the antioxidant properties of the final product **(Şengül et al., 2023)**.

6.2.2.4. Fatty acids

Following the evaluation of the previous components, the fatty acids profile of the chestnut mixtures was determined through CG-FID. A total of twelve fatty acids in the lipid fraction were determined, belonging to three main groups; SFAs, MUFAs, PUFAs **(Table 18)**.

The results showed that PUFAs were the most prevalent fatty acids group in the control mixture with a proportion of 49.72±0.33 % mainly attributed to linoleic acid (C18:2n6c), which presented a percentage of 41.39±0.27 %. In contrast, the PUFAs group was not detected in mixture 1, while mixture 2 presented only a very low percentage (1.42±0.03%).

In contrast, the enriched chestnut mixtures were strongly dominated by SFAs over MUFAs, with the highest percentage being observed in mixture 2 (65.71±0.52%), compared with the lower value observed in the control mixture (16.99±0.11%). Among SFAs, palmitic acid (C16:0) was the most relevant compound identified in all three mixtures, reaching high percentages in mixture 2 (52.90±0.33%) and mixture 1 (46.49±0.55%). Stearic acid (C18:0) was only present in mixture 1, with lower fractions. Minor SFAs including myristic acid (C14:0) and pentadecanoic acid(C15:0) were detected in small amounts, contributing to the overall fatty acid composition.

In addition, mixture 1 contained the highest fraction of MFAs (37.20±0.43 %) compared with the other mixtures, which revealed similar but lower percentages. Oleic

acid (C18:1n9c) was identified as the major MUFA found among the three mixtures, reaching 37.21±0.54% in mixture 1.

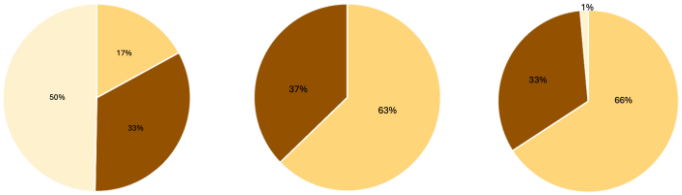
The differences observed among the chestnut mixtures suggest that the decrease in PUFAs and the increase in SFAs after the addition of thermal water may be related to changes in their chemical composition and stability.

Overall, palmitic acid (C16:0) is the most abundant SFA in the enriched chestnut mixtures, whereas the unsaturated fatty acids were predominantly represented by oleic acid (C18:1n9c), which is recognized as an important component of the cell membrane and cell nucleus and supports the absorption of fat-soluble vitamins, including vitamin E **(Ciucure et al., 2022)**.

Despite the variations observed after thermal water enrichment, the fatty acid composition may still support the potential health benefits of the final product.

Table 18: CG-FID Lipidic fraction and fatty acids composition of different mixtures.

	Fatty acids (%)		
Compound Name	**Control Mixture**	**Mixture 1**	**Mixture 2**
C6:0	0.230±0.004^{c}	6.04±0.03^{b}	8.5±0.2^{a}
C8:0	n.d.	1.40±0.01^{b}	2.15±0.02^{a}
C12:0	0.110±0.001^{b}	0.655±0.001^{a}	n.d.
C14:0	0.215±0.004^{c}	0.65±0.01^{b}	0.88±0.01^{a}
C15:0	0.1656±0.0005^{c}	1.009±0.011^{b}	1.48±0.02^{a}
C16:0	16.29±0.12^{c}	46.49±0.55^{b}	52.90±0.33^{a}
C16:1	0.4076±0.0005	n.d.	n.d.
C18:0	nd.	6.63±0.03	n.d.
C18:1n9c	32.91±0.24^{b}	37.21±0.54^{a}	32.98±0.58^{b}
C18:2n6c	41.39±0.27^{a}	n.d.	1.43±0.03^{b}
C18:3n3	7.50±0.09	nd.	nd.
C22:2	0.74±0.01	nd.	nd.
SFA	16.99±0.11^{c}	62.88±0.49^{b}	65.71±0.52^{a}
MUFA	33.34±0.21^{b}	37.20±0.43^{a}	32.7±0.8^{b}
PUFA	49.72±0.33^{a}	n.d.	1.42±0.03^{b}



Relative to fatty acid identification: Palmitic acid (C16:0); stearic acid (C18:0); oleic acid (C18: 1n9c); linoleic acid (C18:2n6c); α-linolenic acid (C18:3n3); Saturated fatty acids (SFA); Monounsaturated fatty acids (MUFA); Polyunsaturated fatty acids (PUFA). For each table row, significant differences with α = 0.05: p < 0.001 between the samples are represented by different letters. nd - not detected.

6.2.2.5. Minerals

After analyzing the chemical composition of the chestnut mixtures, the mineral profile was assessed through AAS and results expressed as relative percentages **(Figure 13)**.

Eight minerals were identified, after thermal water enrichment, namely sodium (Na) potassium (K), magnesium (Mg), iron (Fe), copper (Cu), manganese (Mn), aluminum (Al) and nickel (Ni), revealing differences among the three analysed mixtures.

Referring to the graphical representation, potassium was the major mineral in all chestnut mixtures, presenting the largest proportion of the total mineral content, particularly in the thermal water mixtures with mixture 1 appeared to present the highest relative proportion of potassium, although potassium remained the predominant mineral in all formulations.

A major difference between the control and the enriched mixtures was the presence of sodium. Sodium was not detected in the control mixture and was only identified in mixtures 1 and 2 following thermal water addition. Consequently, sodium became the second most abundant mineral in the enriched mixtures, although at considerably lower proportions compared to potassium. These results strongly suggest that the sodium detected in mixtures 1 and 2 originated from the thermal water used during formulation. According to **Mourelle et al. (2024)**, sodium is an important nutrient required for metabolism, but in low amounts, in order to maintain fluid balance and contribute to proper muscle and nerve functions, and thermal water may contain relevant amounts of sodium, which supports the present findings

Magnesium was identified as the third most predominant mineral, presenting lower proportions compared with both potassium and sodium. Nevertheless, magnesium remained an important contributor to the mineral profile of the enriched mixtures.

The remaining minerals, including Fe, Cu, Mn, Al and Ni were identified in much lower amounts, especially nickel, which presented the lowest relative proportions, as showed by their minimal representation in the graphical profile. Among these lower elements, iron and manganese appeared to contribute more markedly than copper, aluminum and nickel to the overall mineral composition.

Moreover, the amounts of aluminum appeared to increase with the addition of thermal water.

These differences observed among mixtures may therefore be attributed to the effect of thermal water enrichment, particularly due to its mineral composition. In addition to the introduction of sodium, thermal water may also have contributed to changes in the relative distribution of the other minerals in the final products. In fact, thermal water contains relevant amounts of sodium, which may explain the presence of this element in the enriched mixtures, as reported **(Mourelle et al., 2024).**

Overall, the present findings are in agreement with the nutritional profile of the mixtures. Indeed, the ratio of the mineral components (Na:K) represents an important indicator of nutritional potential. In this case, low sodium and high potassium diets interact synergistically to promote the decrease of blood pressure and reduce the risk of arterial hypertension. A balanced mineral composition is important to maintain good health and minimize the risk of diseases **(Musilová et al., 2024; Razzaque & Wimalawansa, 2025)**.

Additionally ,the present results suggest that thermal water enrichment enhances the mineral composition of the final product ,especially by maintaining high levels of essential minerals such as potassium ,sodium and magnesium, which are important for several physiological functions **(Razzaque & Wimalawansa, 2025)**.

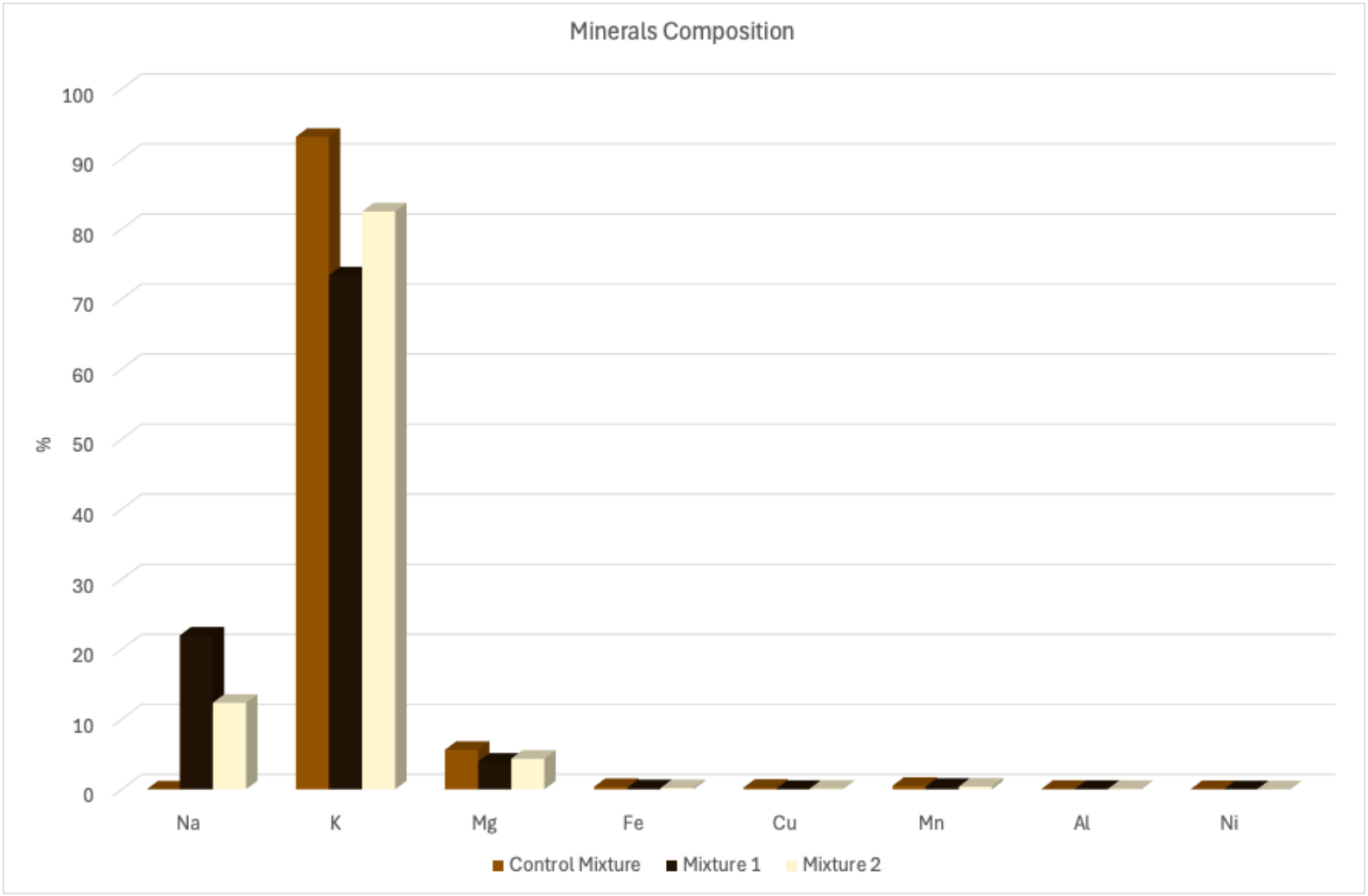


Figure 13*:* Mineral contents of the different chestnut mixtures expressed in percentages.

7. Conclusion

This study demonstrated the potential for valorisation non-commercial chestnut kernels of *Castanea sativa*. Mill from Portugal through the development of freeze-dried chestnuts-based formulations enriched with thermal water. The obtained results confirmed that chestnuts possess a relevant nutritional composition, characterized mainly by high carbohydrate content, moderate protein levels and low lipid content, supporting their importance as a natural source with potential nutritional and technological applications.

The chemical characterization revealed the presence of several compounds of nutritional relevance, including soluble free sugars, tocopherols, organic acids, fatty acids and essential minerals. Sucrose was identified as the predominant soluble sugar, contributing to the characteristic sweetness and sensory quality of chestnuts. The lipid fraction was mainly characterized by the predominance of unsaturated fatty acids, particularly linoleic and oleic acids, which are associated with beneficial physiological effects. In addition, potassium and magnesium were identified as the major minerals, reinforcing the nutritional value of chestnuts as a source of essential micronutrients.

Among the analysed varieties (Amarelal, Judia, Boa Ventura and Longal), Judia presented the most distinctive nutritional profile, particularly regarding carbohydrate, protein, sugar, tocopherol and mineral contents, while Boa Ventura and Amarelal stood out for their higher proportions of unsaturated fatty acids. The observed differences among varieties confirmed the influence of genotype and environmental conditions on chestnut composition.

The enrichment of chestnut mixtures with thermal water positively influenced the mineral composition of the final products, particularly through the incorporation of sodium while maintaining high proportions of potassium and magnesium. Although some variations were observed in the profiles of tocopherols, organic acids and fatty acids after thermal water addition, the final products maintained relevant nutritional characteristics.

Regarding bioactive properties, chestnut fruit extracts did not exhibit significant antimicrobial, antifungal or antiproliferative activities under the tested conditions. Nevertheless, the comparison with previous studies highlighted the importance of

chestnut by-products, particularly shells and burs, as promising sources of bioactive compounds with potential functional and pharmaceutical applications.

Overall, the present work contributes to the valorisation of chestnut by-products and non-commercial kernels by demonstrating their nutritional potential and suitability for the development of value-added food products. Future studies should focus on optimizing thermal water incorporation and exploring the inclusion of chestnut by-products in order to enhance the bioactive potential and functional properties of the developed formulations.

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