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Comparison of nutritional and bioactive properties of acorn flour with and without tannins

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Acknowledgments

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Index

List of tables

Index of Figures

List of abbreviations

Quercus spp : *Quercus* species pluralis

Q: Quercus

SPM21: whole Acorn flour

SPM21F4: acorn flour without tannins

DAD: diode array detection

ESI: electrospray ionization

EtOH: ethanol

GC-FID: gas chromatography- flame ionization detector

HPLC: high-performance liquid

MS: mass spectrometry

MUFA: monounsaturated fatty acid

PUFA: polyunsaturated fatty acid

SFA : saturated fatty acid

TFAs: total fatty acids

Abstract

The *Quercus* spp. are a varied group of evergreen and deciduous trees native to temperate and tropical regions. There are about 600 species of *Quercus* worldwide, which differ in their flowering and fruiting dynamics as well as maturity index. Acorns, the nuts of *Quercus* spp. trees, offer a comprehensive nutritional profile, being rich in proteins, fibers, starch, bioactive compounds including tannins and phenolic acids, essential vitamins, like vitamins A and E, and minerals, such as potassium, iron, calcium, and magnesium. However, this fruit presents a problem for the food industry regarding the astringency caused by tannins, which are anti-nutrients found in acorns. Therefore, this study aims to evaluate the potential of acorn flour for the baking industry by comparing the nutritional value and bioactive compounds of acorn flour with and without tannins. The whole flour (SPM21) and a tannin-free flour (SPM21F4) were analysed, having both been supplied by the company Landratech. The elimination of tannins, which was accomplished by leaching the flour three times with water at 15 °C, resulted in a reduction in several nutrients, such as crude protein, ash, and fiber. However, the SPM21F4 flour showed increased levels of moisture, crude fat, starch, total carbohydrates, and energy content. Soluble sugars were found exclusively in the SPM21 flour, indicating that the leaching treatment affected the sugar content in the SPM21F4 sample. The fatty acid profile of the SPM21F4 flour revealed higher levels of polyunsaturated fatty acid (PUFA) and monounsaturated fatty acid (MUFA), while the flour containing tannins had higher levels of saturated fatty acids (SFA). Ten phenolic compounds were detected, and the tannin free flour had a much lower overall phenolic content. In conclusion, properly processed acorn flour can improve the nutritional profile of bread by providing bioactive chemicals and appealing sensory features, hence increasing its popularity in the baking business. However, further research is needed to optimize tannin extraction and fully understand the nutritional and industrial potential of acorn flour.

Keywords: Acorn flour, Tannins, Nutritional value, Phenolic compounds, Soluble sugars, Fatty acids, Baking industry, Tannin removal.

Resumo

As espécies do género *Quercus* são um grupo variado de árvores perenes e decíduas nativas de regiões temperadas e tropicais. Existem cerca de 600 espécies de *Quercus* em todo o mundo, que diferem nas suas dinâmicas de floração e frutificação, bem como no índice de maturidade. As bolotas, os frutos das árvores deste género, oferecem um perfil nutricional abrangente, sendo ricas em proteínas, fibras, amido, compostos bioativos, incluindo taninos e ácidos fenólicos, vitaminas essenciais, como as vitaminas A e E, e minerais, como potássio, ferro, cálcio e magnésio. No entanto, este fruto apresenta um problema para a indústria alimentar devido à adstringência causada pelos taninos, um anti nutriente encontrado nas bolotas. Este estudo visa avaliar o potencial da farinha de bolota na indústria de panificação, comparando o valor nutricional e os compostos bioativos da farinha de bolota com e sem taninos. Foram analisadas farinhas com presença de taninos (SPM21) e uma farinha sem taninos (SPM21F4), sendo ambas as amostras fornecidas pela empresa Landratech. A eliminação dos taninos, realizada através da lixiviação da farinha três vezes com água a 15 °C resultou na redução de vários nutrientes, como proteína bruta, cinzas e fibras. No entanto, a farinha SPM21F4 apresentou um aumento nos níveis de humidade, gordura bruta, amido, carboidratos totais e conteúdo energético. Açúcares solúveis foram encontrados exclusivamente na farinha SPM21, indicando que o tratamento de lixiviação afetou o conteúdo de açúcar na amostra SPM21F4. O perfil de ácidos gordos da farinha SPM21F4 revelou níveis mais altos de ácidos gordos polinsaturados (PUFA) e monoinsaturados (MUFA), enquanto a farinha com taninos apresentou níveis mais altos de ácidos gordos saturados (SFA). Dez compostos fenólicos foram detetados, e a farinha sem taninos apresentou um conteúdo fenólico total muito menor. Concluindo, a farinha de bolota adequadamente processada pode melhorar o perfil nutricional do pão, fornecendo compostos bioativos e características sensoriais aliciantes, aumentando assim a sua popularidade na indústria de panificação. No entanto, serão necessários mais estudo de forma a otimizar a extração de taninos e entender completamente o potencial nutricional para a industrial de farinha de bolota.

Palavras-chave: Farinha de bolota, Taninos, Valor nutricional, Compostos fenólicos, Açúcares solúveis, Ácidos gordos, Indústria de panificação, Remoção de taninos.

1. Introduction

1.1. Baking industry

1.1.1. Importance of this industry in the world

The flour and baking industry has a high significance in the global food sector, playing a pivotal role in providing essential products that are integral to diets worldwide. The product of ground grains, flour, is the base ingredient for a wide range of food items, including bread, pastries, cakes, and other baked goods. The substantial economic size and steady growth of this sector emphasize its significance. There was a notable compound annual growth rate of 6.8% in the global bread and bakery products market, which grew from $226.25 billion in 2022 to $241.69 billion in 2023 [1].

The wheat flour market is being driven forward by the growing demand for ready-to-eat food and bakery products [2]. The increasing popularity of convenience foods and urbanization are some of the factors that influence this demand. The baking, pasta, and biscuit industries are especially important for ensuring food availability, as they aid in tackling global issues, such as a growing population, environmental changes, and the need for eco-friendly resource administration [3].

The impact of the flour industry extends beyond the food and beverage industry, playing an important role in a variety of sectors including food service establishments, baking, pastry making, pasta and noodle production as well as snack production. Its versatility is evident not only in the culinary sector but also in non-food industries, at pharmaceuticals and especially cosmetics [4]. There are some researches that explores the extraction and modification of jasmine rice powder as an alternative to talcum powder, a common but potentially harmful ingredient in powder compacts, using the polyethylene glycol (PEG)50 shea butter to improve the physical properties of rice flour, focusing on improving fluidity and water resistance. This rice powder modification appears to be a safer and more effective alternative to talc in compacted powder formulations, demonstrating superior properties such as sun protection factor (SPF) over 15, ultraviolet B (UVB) protection, excellent coverage, natural appearance, skin softness, diffusion and increased user satisfaction compared to commercial products [5].

The Asia-Pacific region leads the global wheat flour market, with China and India dominating the consumption of flour.

The flour and baking sector not only holds economic significance but also plays an essential part in ensuring food safety. By supplying essential foods consumed daily worldwide, this industry plays a crucial role in meeting the evolving needs and challenges of today's world [6-8].

1.1.2. Baking industry in Portugal

The flour and baking industry in Portugal holds cultural and economic significance. In the region surrounding Lisbon, heritage grains such as Saloia wheat flour have been revived, a testament to their historical and cultural importance. A revival in bread production has been influenced by Portugal's political and economic history [9]. The adoption of healthier eating habits by Portuguese consumers is shaping innovation in the bread market with a focus on nutritionally superior flours and healthier options [10]. The sector's financial impact goes beyond its market value, supporting agriculture, milling, flour packaging, transportation, and retail enterprises, and is expected to continue expanding [4]. The sector of spelt and baked goods in Portugal is, despite the obstacles, an essential component of the nation's culinary heritage and economic life.

Figure 1 demonstrates that in 2024, the Bread & Bakery Goods Production industry in Portugal is valued at €1.5 billion and is ranked 11th Europe, representing its revenue for the year. However, the industry has experienced a decline over the past four years, with the market size decreasing by an average of 1.4% annually from 2021 to 2024.

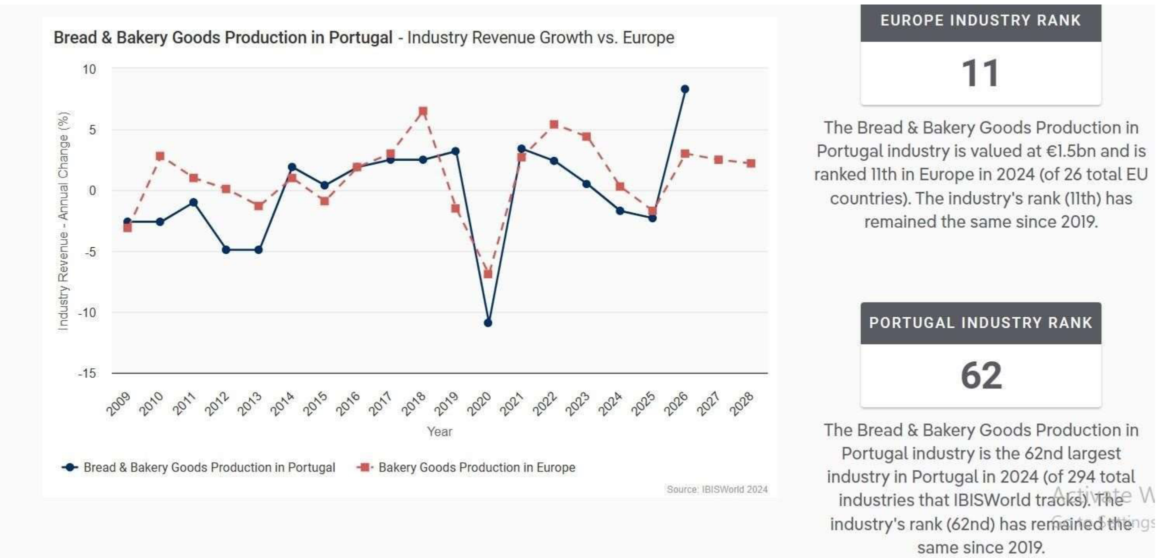

Figure 1. Bread & bakery goods production in Portugal - industry statistics 2009–2026 [11].

1.2. Acorn

The *Quercus* spp. are a varied group of evergreen and deciduous trees native to temperate and tropical regions. There are about 600 species of *Quercus* worldwide, which differ in their flowering and fruiting dynamics as well as maturity index. These fruits resemble acorns, which have found recognition globally [12-13].

Acorns are characterized by a single-seeded nut with no endosperm and an achlorophyllous embryo morphologically; they are universally regarded as nutrient-dense products, justifying their usage as secondary human diets (mostly supplies of carbohydrates, proteins, and fat) or food additives, for thousands of years wherever oak trees can be found [14]. A thorough consideration of acorns and their constituents may enhance their value as potential materials for future use in food or pharmaceutical industries due to their nutraceutical, phytochemical, and bioactive properties [15]. The Mediterranean basin's forests and woods have various kinds of architecture, appearance, and woody plant composition due to differences in ecological conditions as well as land use history. In the meso- and supra-Mediterranean life zones, oak trees are the most dominant species.

In northern Portugal, the major forms of these are pure woodland consisting of *Quercus robur* and *Quercus pyrenaica* and mixed woodlands.

Primarily found in the western Mediterranean basin is evergreen cork oak (*Quercus suber* L.). The largest forest cover that is estimated at nearly 700,000 ha are situated in Portugal covering 21% of its forest area but also occupying 30% of world cork producing areas [16]. However, there has been reports that *Q. suber* hybridizes with some other *Quercus* species such as *Q. ilex* which constitutes part of *Quercus ilex* L. complex thus contributing to enhanced genetic diversity in cork oak [17].

Table 1 presents the most common *Quercus* species found in different parts of the world. Figure 2 shows some photos of trees of the *Quercus* species most abundant in mainland Portugal.

Table 1. Different types of Quercus from different sources [18].

Source	Acorn species
Turkey	*Q. pontica* *Q. robur* *Q. hatwissiana* *Q. frainetto* *Q. petraea* *Q. vulcanica* *Q. ithaburensis* *Q. brantii* *Q. libani* *Q. trojana*
Spain	*Q. faginea* *Q. suber* *Q. pyrenaica* *Q. coccifera* *Q. ilex*
Portugal	*Q. faginea* *Q. ilex* *Q. nigra* *Q. suber*
Algeria	*Q. ilex* *Q. suber*
Jordan	*Q. aelgilops* *Q. infectoria* *Q. calliprinos*
Latvia	*Q. rubra* *Q. robur*
Serbia	*Q. robur* *Q. cerris*

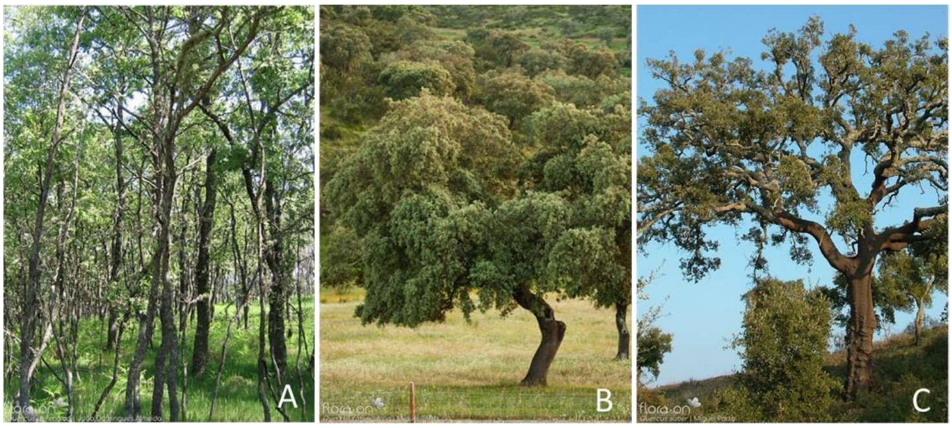

Figure 2. Different species of *Quercus*, **A**: *Quercus pyrenaica*; **B**: *Quercus rotundifolia*; **C**: *Quercus suber* [19-20].

1.3. Acorn morphology

An acorn is a 1-seeded nut, known by the absence of an endosperm and the presence of an achlorophyllous embryo (Figure 3). It is composed of a cupule (the cup), pericarp (the fruit) and the seed coat (or shell for protection) [14-15].

In general, phylogenetic, and ecological factors are responsible for differences among acorns of *Quercus* species. There is great variability in seed size within and between oak species and it has been proved that acorn size is positively correlated with the length of its development period and with rainfall [21]. However, the size of a fully developed acorn always depends on its growth conditions. Therefore, an acorn size is also positively connected with seedling survival rate under stress conditions [14-15].

Figure 4 shows some photos of the different acorns originating from the most abundant species of *Quercus* present in mainland Portugal.

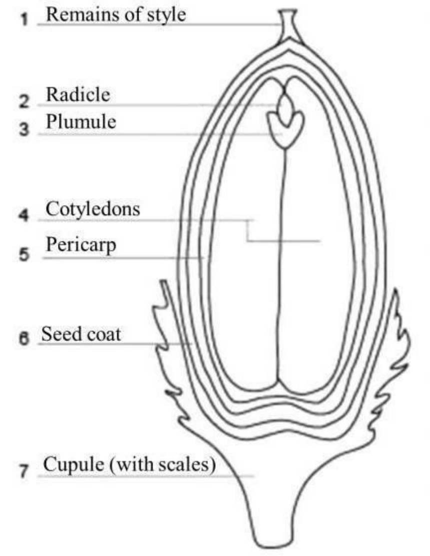

Figure 3. Acorn sketch highlighting the main morphological characters [14].

Figure 4. A: *Quercus ilex*; **B**: *Quercus rotundifolia*; **C**: *Quercus suber*; **D**: *Quercus pyrenaica* **[**22].

1.3.1. Importance of acorn

Acorns, the nuts of oak trees, have recently become the focus of numerous scientific studies, revealing their noteworthy nutritional value, health benefits, and environmental significance. Acorn offers a comprehensive nutritional profile, being rich in essential vitamins, proteins, fibers, and minerals, including tannins, flavonoids, vitamins A and E, potassium, iron, calcium, and magnesium.

The diverse array of nutrients present in acorns contribute to a range of health benefits, including improved digestion, heart and bone health, and reduce the risks of asthma and diabetes mellitus. Notably, acorns also possess antimicrobial and antiviral properties, enhancing their potential health-promoting qualities.

Beyond their health benefits, acorns have also found numerous culinary uses, appearing like acorn flour, acorn brittle, acorn squash, and acorn jelly. Acorns are recognized as a common, eco-friendly and sustainable food source, especially in the fall, and they are a crucial resource for the survival of animals. Recognizing the presence of bitter and astringent tannins in acorns is crucial.

Proper preparation methods, such as boiling or soaking, are suggested to improve the taste of these compounds. In conclusion, acorns emerge as a nutritious food source with significant health advantages and a beneficial effect on the environment. The extensive scientific research dedicated to understanding their nutritional value and potential uses underscores their importance as a valuable and versatile natural resource [23-27].

1.3.2. Uses in several industries

Food production, leather processing, animal feed, and tanneries are some of the industries where acorns are used. The essential amino acids, fatty acids, vitamins, minerals, and polyphenols in acorns make them an excellent source of nourishment and useful. compounds. Researchers have suggested several uses for acorns, including the production of bioethanol, biobutanol, biodiesel, fuel briquettes, activated carbon, and natural coagulants. Acorns can be used as an absorbent for hexavalent chromium removal, and for the extraction of bioactive compounds like carotenoids, tocopherols, phytosterols, and phenolic compounds. The popularity of the cold-pressed method for oil extraction from plant materials seems to favour the extraction of oil from acorns for small-scale. applications. The processing industry has a lot of potential for using acorns for cheap plant material. Acorns are used in a variety of industries, and their potential for further applications is still being explored [28-31].

1.3.3. Usage in the food industry

The food industry has discovered that acorns have numerous uses, and their potential for further applications is still being explored [32-34]. The starch, essential amino acids, fatty acids, vitamins, minerals, and polyphenols in acorns make them a valuable source of nourishment and functional compounds [35].

Acorns are also used as feedstuff, resulting in meat products of high quality as it is rich in vitamins and minerals, have been a staple food for pigs, and, in recent years, the practice of filling pigs with acorns has gained traction even in countries that don't typically practice it. Fat content and flavour profile of pigs finished with acorns reflect the unique flavour and fat profile [36].

Overall, acorns have a wide range of uses in the food industry, and their potential for further applications is still being explored [37].

1.4. Tannins

1.4.1. Tannins and their role in nature

Natural polyphenolic compounds called tannins are widely distributed in the plant kingdom and play an important role in nature. Tannins can be found in various parts of plants, such as leaves, bark, fruits, and seeds, and they serve a variety of purposes, including defense against herbivores, protection against pathogens, and control over plant growth and development [38, 39]. There are several subclasses of tannins. In Figure 5 it is possible to observe the different groups of existing tannins as well as an example of a compound belonging to each of these groups.

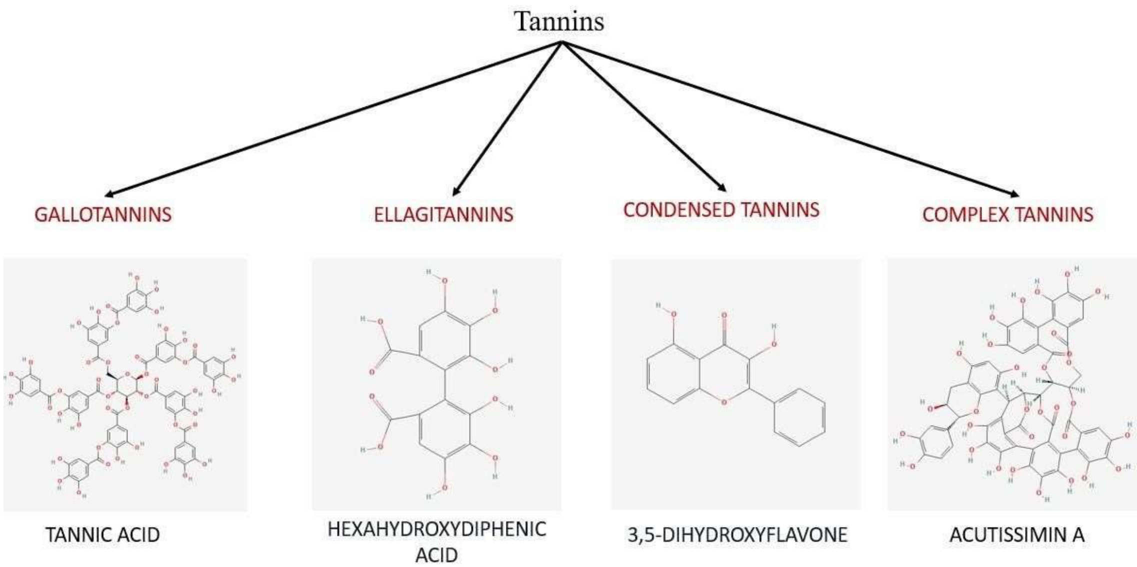

Figure 5. Classification of different groups of tannins.

Acorns are extremely rich in tannins, being used for centuries in leather treatment and tanning. The leather industry can use acorn tannins because they have excellent tanning properties. There are meat products of high quality and traditional leather tanning made from acorns, which are used as feedstuff. Acorns have been found to have several health benefits, such as anti-inflammatory, anti-cancer, and anti-diabetic properties [40].

1.4.2. Industrial applications of tannins

Tannins play a significant role in many industries, including the tanning industry, medical, pharmaceutical, and food industries. The tanning industry commonly uses acorns because of their excellent tanning properties. The medical and pharmaceutical industries can use tannins due to their antimicrobial, antioxidant, and anti-inflammatory properties. They are also capable of functioning as a pesticide and nutritional supplement. The food industry uses tannins as a natural food preserver and as a source of natural pigments. Tannins also hold promise as a raw material for environmentally friendly industries, such as the production of biodiesel and bioplastic. The potential world supply of tannins is substantial, and future applications are still being developed, promising to have an impact on several industries. However, the supply of tannins is limited, and additional factories are needed to extract them. Further progress is needed to prove the effectiveness of medical and pharmaceutical applications in vivo [41].

1.4.3. Tannin removal in nuts

Although tannins show high potential to be applied in several industries, their consumption in foods can also lead to causing gastrointestinal issues and interfering with nutrient uptake, as well as giving astringent taste to some foods. The latter has been one of the reasons acorns and their flour has not been widely introduced in foods. It is therefore essential to remove tannins from these nuts to facilitate their use in the food industry. This removal can be done through soaking, boiling or roasting. The most common method used to remove tannins from nuts is to soak them in water for several hours or overnight. Tannins can also be removed from nuts by boiling them in water or by roasting. The roasting procedure can also enhance the nutty taste. It is essential to remove tannins from nuts to ensure their safety and to prevent any negative health effects [42].

1.4.4. The effects of tannins on the nutritional value of nuts

The phenolic compounds in nuts can affect their nutritional value. Tannins can cling to proteins and minerals in nuts, reducing their availability for digestion by the body. The chelation of minerals by tannins can lead to mineral deficiencies, such as iron deficiency. By soaking, boiling, and roasting, it is possible to reduce the negative effects of tannins on the nutritional content of nuts. The tannins in processed legume seeds are reduced by soaking and hydrothermal treatment, enhancing their accessibility to protein and mineral components. Eliminating tannins from nuts is crucial for ensuring their security and avoiding any adverse health consequences associated with ingesting tannins [43-45].

2. Objectives

The main objective of this work is to compare the differences between flours made from acorn nuts with flour made from acorns without tannins focusing on:

- Comparing the nutritional profile of both flours, namely the moisture, protein, crude fat, ash, fiber, starch, carbohydrates, and energy.
- Comparing the individual molecules between the flours, including the phenolic compounds, fatty acids and soluble sugars.

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3. Methodology

To evaluate the nutritional composition and the bioactive properties of the acorn flour, two different groups of laboratory tests were carried out. These tests aimed to obtain the centesimal composition (moisture, protein, fat, ash, fiber, starch and energy), nutritional composition (soluble sugars and fatty acids) and bioactive compounds characterization (phenolic compounds).

The study used two different acorn flour samples, identified as SPM21 (untreated flour) and SPM21F4 (treated flour) (Figure 6).

Figure 6. Representative image of the 2 samples analyzed. On the left, the untreated sample (SPM21) and on the right, the sample treated to reduce tannins (SPM21F4).

Both samples belong to the species *Quercus suber* and were collected from Paradela, Mogadouro, Bragança. These samples were provided by the company Landratech and this study was made in the aim of the project "MEDACORNET - Resgate de bolotas como um Superalimento Tradicional Mediterrânico (PRIMA/0005/2022)". To make acorn flour, the shell of the acorns was removed, and the pulp was ground using a disc mill (Oukaning, China). While the SPM21F4 sample was subjected to three separate leaching treatment at 15 ºC to extract tannins, the SPM21 sample did not receive any treatment. The samples were dried at a constant 50 ºC temperature. Table 2 shows all relevant information regarding the samples.

Table 2. Characteristics of the two samples used in the study.

Samples	SPM21	SPM21F4
Type of flour	Acorn flour	Acorn flour without tannins
Dry temperature	50 ºC	50 ºC
Origin	Paradela, Mogadouro, Bragança	Paradela, Mogadouro, Bragança
Species	*Quercus suber*	*Quercus suber*
Treatments	No treatment	Leaching 3x at 15 ºC

3.1. Proximate Composition and Chemical Characterization

3.1.1. Proximate composition

To determine the centesimal composition of the flour samples, standard methods were applied according to AOAC, 2023 [46]. For the crude protein content, a conversion factor was used, following AOAC recommendations (N × 5.3), for the Macro-Kjeldahl method. Crude was extracted with petroleum ether in a Soxhlet equipment. Ash content was calculated by incineration in muffle at 550 °C. The fiber content was determined using a total dietary fiber enzymatic assay kit. The total carbohydrates were calculated by difference, applying the equation: Carbohydrates (g/100g) = 100 – (moisture (g/100g) + protein (g/100g) + fat (g/100g) + ash (g/100g)). Finally, the total energy value was calculated using the equation: Energy (kcal) = 4 × proteins + 9 × lipids + 2 × fiber + 4 × (total carbohydrates – fiber).

To determine the starch content of the acorn flours, the official AOAC 996.11 (Neogen Corporation & Megazyme Ltd., 2023) method and the protocol was used. This method involves weighing the crushed sample (~100 mg) into a Corning culture tube. After adding 0.2 mL of aqueous ethanol (80% v/v) to aid dissolution, 3 mL of the thermostable αamylase enzyme diluted 30x in sodium acetate buffer (200 mM, pH4.5) were added and the tube incubated in a boiling water bath for 6 minutes. Then, the tube was transferred to a bath at 50 °C, where 4 mL of buffer were added followed by 0.1 mL of the enzyme amyloglucosidase.

After incubation for 30 minutes, the contents were transferred to a 100 mL volumetric flask and the volume was gauged with buffer. Aliquots of 2 mL were collected into eppendorfs and the contents are centrifuged at 1000 g for 10 minutes. After this step, 0.1 mL were transferred to glass test tubes where the glucose oxidase/peroxidase reagent (GOPOD) was added. The test tubes were incubated at 50 °C for 20 minutes, and the absorbance was measured at 510 nm to calculate the starch content. The results were expressed in g per 100g of dry weight.

3.1.2. Soluble sugars

The quantification of soluble sugars was done using high-performance liquid chromatography coupled with a refractive index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany), using melezitose as an internal standard. The analytical procedures outlined by Mandim et al. 2022 [47] were followed. Therefore, the acorn flours, the samples were extracted with a aqueous ethanol (EtOH:H2O, 80:20 v/v, 40 mL) and the internal standard melezitose (IS, 5 mg/mL) at 80 ºC for 90 min. The solution was centrifuged at 15,000g for 10 min and the supernatant was concentrated under reduced pressure (rotary evaporator Büchi R210, Flawil, Switzerland). The obtained solution was defatted with ethyl ether (10 mL, three times) and the solid residues were dissolved in water to a final volume of 5 mL. The soluble sugars content was analysed by High Performance Liquid Chromatography (HPLC, Knauer Smartline 2300, Knauer, Berlin, Germany), coupled to a refractive index detector (RI detector, Knauer Smartline 2300, Knauer, Berlin, Germany) following the procedure previously described (Dias et al., 2015). The identification was performed through the comparison of the retention times with the commercial standards (D-(−)-fructose, D-(+)-sucrose, D-(+)-glucose, D-(+)trehalose, and D-(+)-raffinose pentahydrate purchased from Sigma-Aldrich (St. Louis, MO,USA). For the quantification, the areas of the peaks were compared with the calibration curves of the commercial standards. The results were analysed using the Clarity 2.4 software (DataApex, Prague, Czech Republic) and were expressed in g per 100 g of dry weight.

3.1.3. Fatty acids

Following Soxhlet extraction, the samples were transesterified in accordance with the procedure reported by Albuquerque et al. 2023 [48]. Gas chromatography (GC-FID, DANI 1000, Milan, Italy) was used for analysis. Fatty acid identification was done by comparing the relative retention times of the FAME (fatty acid methyl esters) peaks with those of standard samples (standard mixture 47885-U, Sigma, St Louis, USA). The obtained results were processed using the Clarity 4.0.1.7 Software (Informer Technologies, Inc., Solihull, Great Britain) and expressed as relative percentages (%).

3.3 Bioactive Compounds Characterization

3.3.1 Phenolic Compounds

To carry out the phenolic compound analysis, the samples underwent an aqueous extraction process with a solid/liquid ratio of 25 g/L for 1 hour at 40 °C with constant stirring. After filtration, the samples were freeze-dried (FreeZone 4.5, Labconco, Kansas City, MO, USA), and the studied acorn shell extracts were obtained.

After obtaining a lyophilized extract, it was dissolved in an ethanol-water solution (20:80, v/v) to achieve a final concentration of 10 mg/mL. Subsequently, the solution was filtered through a syringe filter, as described by Madureira et al. 2023 [49]. Detection and quantification were done using a liquid chromatography (HPLC-DAD-ESI/MSn) Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system equipped with a diode array detector (DAD) coupled to an electrospray ionization mass detector, a quaternary pump, an auto-sampler (kept at 5 °C) and a degasser and an automated thermostatted column compartment. Double online detection was performed at preferred wavelengths of 280 nm and 370 nm. Additionally, a mass spectrometer (MS) was connected to the HPLC system through the DAD cell outlet for enhanced detection. Compound identification was done by comparing chromatographic parameters with available literature and using available standards. The results were expressed in milligrams per gram of extract.

3.4. Statistical Analysis

Throughout the whole document, results are shown as mean±standard deviation. For comparison between the full flour and flour without tannins the means were subject to an analysis of variance (ANOVA) followed by a Student's T-test for classification. All statistical operations were performed with a confidence interval of 95%.

4. Results and Discussion

The primary aim of this work was to compare acorn flour with and without tannins. Acorn flours, as well as other nut flours are becoming prevalent as substitutes of cereal flours due to their higher nutritional properties. Still, tannins are molecules that impart astringent flavors to foods and can be removed through several processes. Thus, their removal might change other nutritional or molecular properties of the acorns. Therefore, this work aimed to identify what changes may occur.

4.1. Proximate composition

To provide a thorough comparison between the two types of acorn flours, the analysis began by assaying the nutritional composition for the two samples SPM21 and SPM21F4, such as moisture, crude protein, crude fat, ash, total carbohydrates, fiber, starch and energy content, which are represented in Table 3.

Table 3. Nutritional composition of the two samples.

	Moisture (g/100 g)	Crude protein (g/100 g)	Crude fat (g/100 g)	Ash (g/100 g)	Total Carbohydrates (g/100 g)	Fiber (g/100 g)	Starch (g/100g)	Energy (kcal/100g)
SPM21	9.5 ± 0.5*	6.03±0.03*	5.1 ± 0.3*	2.3 ± 0.2*	77.2 ± 0.5*	27.3 ± 1.0*	20.14 ± 0.09*	324 ± 2*
SPM21F4	10.2 ± 0.6	5.3±0.1	5.6 ±0.1	0.90 ± 0.09	78.0 ± 0.7	19.0 ± 0.6	54.2 ± 0.6	345 ± 3

Results are expressed in dry weight; each value is the mean ± standard deviation; an asterisk (*) means a significant difference between each sample with a significance of 0.05

As it can be seen in Table 3, there were statistically significant differences between the two samples. In the case of SPM21 (whole sample), it contained 6.03 g/100g of crude protein, while the SPM21F4 (sample without tannins) samples showed only 5.3 g/100g. Inversely, regarding moisture and crude fat, the sample without tannins (SPM21F4) showed higher amounts, namely 10.2 and 5.6 g/100g respectively, while the sample without treatment (whole sample) registered 9.5 and 5.1 g/100g, showing a statistically significant difference, thus the ranges are very similar. Fiber and ash also reduced in the sample without tannins, with statistical significance. Interestingly, starch content increased to slightly more than double in SPM21F4 flour compared to untreated flour. This interesting fact may be due to the leaching process used in sample SPM21F4, hence the main purpose of leaching is to remove tannins. While doing so, some soluble carbohydrates and other nutrients may also be washed, but the starch, being less soluble in cold water, mostly remained. The process might indirectly result in a higher relative concentration of starch in the final flour if a significant amount of non-starch components were removed. Summarizing, the leaching process with cold water does not increase the actual amount of starch in acorn flour. Instead, it helps to remove tannins (which cause astringent flavor to the flours) and other soluble substances, potentially increasing the relative proportion of starch by weight in the final product after drying. The energy ended up increasing in sample SPM21F4, which was expected due to the starch discrepancy found between the 2 samples.

4.2. Fatty acids

Table 4 shows the three soluble sugars found in the two samples of acorn flour. As it can be seen, these sugars were only found in the SPM21 flour, so it can be inferred that the treatment applied to the sample SPM21F4 affected the sugar content. That is due to the solubility of the sugars which can be inferred that they were drawn out of the flour during the leeching process, thus not showing in the SPM21F4 sample.

Table 4. Soluble sugars detected and quantified in the full acorn flour, expressed in g/100g of dry weight.

Compound Name	Retention Time [min]	Amount [g/100g]
Fructose	8.327	4.23±0.06
Glucose	9.157	3.9±0.4
Sucrose	13.033	10.8±0.4
	Total	18.9±0.8

The most abundant soluble sugar in the flour was sucrose with 10.8 g/100g, followed by fructose, being glucose the least abundant. Together, the soluble sugars amassed 18.9 g/100g of dry weight of the full flour.

Figure 7 shows an example chromatogram of the quantification of the different soluble sugars in sample SPM21 (the only sample that presented quantifiable soluble sugars). In this chromatogram it is possible to see the 3 peaks relating to the identified sugars, as well as the peak relating to the internal standard used (melozitose).

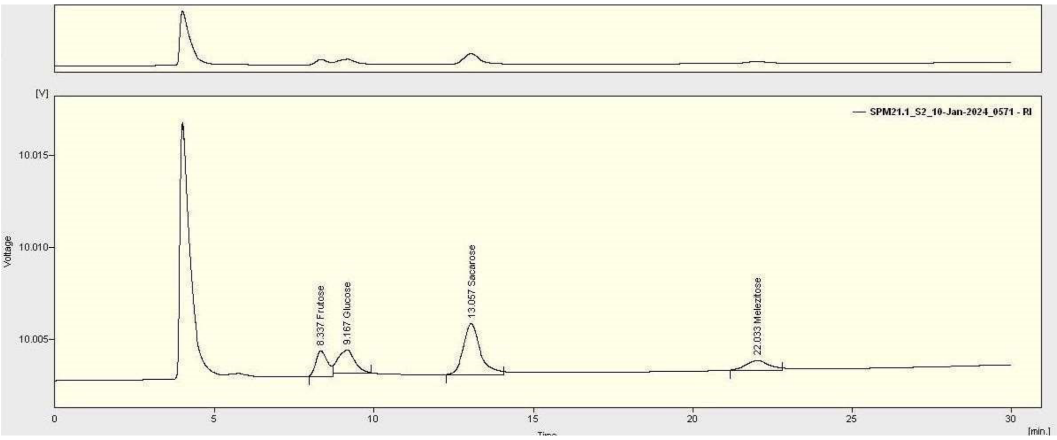


Figure 7. Chromatogram of the quantification of the different soluble sugars in sample SPM21.

Overall, the variations shown in the two flours could be due to a removal of some nutrients by the leaching process, which removed some nutrients. The increase in moisture in the samples without tannins could be due to some water remaining in the sample also from leaching.

Comparing these results with literature, it could be mentioned that they are similar to those previously reported by Galván et al. 2011 [50], in which they studied Holm oak (*Quercus ilex* subsp. ballota) by analysing the acorn protein profile in flour through SDSPAGE and mass spectrometry. Özcan et al. 2006 [51] studied the total protein and amino acid compositions in mature acorns of 20 *Quercus* taxa from Turkey, in which *Quercus ilex* was evaluated, finding that it had the lowest protein content on average (3.35%) compared to *Quercus* cerris (4.22%) and other *Quercus* species (5.11%). Regarding moisture, the results obtained are in agreement with literature, namely Amina et al 2018 [52], that found levels of moisture of 10.37% in the flour, which were higher than the ones found by Rakic et al. 2006 [53], at 7.89% for *Quercus robur* and Li et al. 2015 [54] for *Quercus glandulifera* flour at 7.55%. Regarding fat, acorn flour shows the highest amount when compared with other common flours, such as rice (0.90 g), wheat (1.81 g), maize (2.48 g), sorghum (3.50 g), wholewheat (3.63 g), buckwheat (4.21 g), or oat (6.74 g), according to Martins et al.2022 [55]. Regarding fat, the slight difference between SPM21 and SPM21F4 indicated that tannins can bind fat molecules and make them less measurable in standard nutritional analyses.

Ojo, 2022 [43] found that the presence of tannins can indeed affect the measurability of fat content in food by creating complexes with fat molecules. In terms of ash, in literature, the ash content for *Quercus suber* is an average of 2.30 g/100 g according to Larotonda et al. 2007 [56] and 1.9 g/100g according to González et al. 2006 [57], which are in total agreement with the results found in this work.

4.3. Fatty acids

Fatty acids are molecules of great interest among the fat of food samples. They are responsible for flavor and specific rheological properties in food. Table 5 presents the detected fatty acids identified in the two flour samples.

Table 5. Fatty acids detected in both flour samples, expressed in relative percentage.

	SPM21	SPM21F4
C14:0	0.27±0.04*	0.245±0.009
C15:0	nd	0.101±0.003
C16:0	18.9±0.6*	18.22±0.105
C17:0	0.126±0.009*	0.154± 0.001
C18:0	2.3±0.3*	2.0965±0.0005
C18:1n9t	3.2±0.3*	1.9945±0.005
C18:1n9c	52.6±0.6*	50.78±0.02
C18:2n6t	0.251±0.006	0.251±0.006
C18:2n6c	17±1*	22.68±0.04
C18:3n3	0.2±0.01*	1.74±0.03
C20:0	0.35±0.05*	0.38±0.02
C20:1	2.2±0.3*	0.497±0.008
C20:2	2.2±0.3	0.24±0.01
C22:0	0.22±0.01	0.326±0.002
C24:0	1.40±0.08	0.283±0.005
MUFA	58.0±0.1*	53.1±0.5
PUFA	17±1*	24.7±0.9
SFA	23.6±0.6*	21.8±0.2

Results are expressed in dry weight. C14:0-Myristic acid, C14:1-Myristoleic acid, C15:0-Pentadecylic acid, C16:0Palmitic acid, C17:0-Margaric acid, C18:0-Stearic acid, C18:1n9t-trans-oleic acid,C18:1n9c-Oleic acid, C18:2n6ttrans-linoleic acid, C18:2n6c-Linoleic acid, C18:3n3-α-linolenic acid, C20:0-Arachidic acid, C20:1Eicosenoic acid, C20:2-Eicosadienoic acid, C22:0-Behenic acid, C24:0-Lignoceric acid. An asterisk (*) means a significant difference between each sample with a significance of 0.05.

A total of 15 fatty acids were detected, corresponding to monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and saturated fatty acids (SFA). Overall, in both flour samples, the most abundant group of fatty acids were the MUFA, followed by SFA in SPM21 and PUFA in SPM21F4. This result is quite interesting as MUFA and PUFA are regarded as healthy fatty acids, while SFA are undesirable fats, although they are high contributors to flavor. While there is a notable and statistically significant decrease in SFA and MUFA from full flour to the one without tannins, this does not happen in terms of PUFA. This could be due to a removal of fatty acids during the leeching process, and being expressed in relative percentage, the removal of preferably MUFA and SFA, makes the PUFA increase in the flour without tannins. Individually, the most abundant fatty acid in both samples was cis oleic acid, with higher prevalence in the full flour. Five individual fatty acids increased their amounts in a statistically significant manner, namely margaric acid, linoleic acid, α-linolenic acid, arachidic acid, and behenic acid. All other fatty acids significantly reduced their quantities from the full flour to the flour without tannins, probably due to the leeching process. Pentadecylic acid was only detected in the flour without tannins, probably due to the breakdown of a MUFA, although only showing about 0.1% of total fatty acids.

Figure 8 shows a chromatogram relating to the quantification of fatty acids in the SPM21F4 sample. Highlighted in different colors are the peaks corresponding to the majority fatty acids found in this sample (Green – oleic acid, Blue – linoleic acid, Red – palmitic acid).

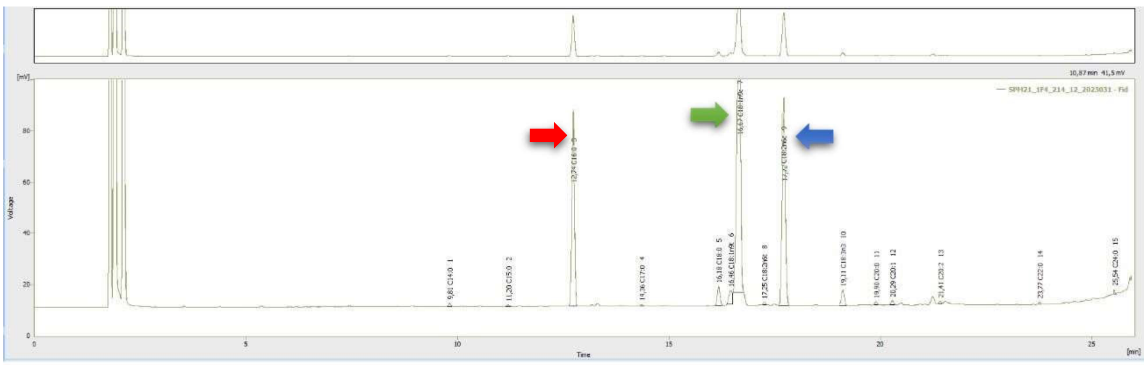


Figure 8. Representative chromatogram of fatty acid quantification for sample SPM21F4.

These findings are aligned with those previously reported by Charef et al. [58] regarding the fatty acid composition of *Q. ilex* and *Q. suber* acorn species grown in Algeria, as well as by Papoti et al. [26] in their study of products and by-products derived from acorns. In the latter study, Papoti et al. noted that the predominant fatty acid in all samples was oleic acid (C18:1n9), which accounted for about 50%, followed by palmitic (C16:0) and linoleic (C18:2n6) acids, each representing about 23% of the total fatty acids detected. Additionally, this study and Papoti et al, both observed slight increase in PUFA content in samples that underwent the leaching process, which reinforces that this process increases, albeit in a small amount, the concentration of PUFA present in the leached samples.

In another study, Akcan et al. 2017 [59] reported the same fatty acids in there study of the acorn *Quercus suber* as a novel source of oleic acid and tocopherols for livestock and humans finding the most abundant fatty acid was C18:1n9 (56.25%), which is similar of the percentage found in our samples, also detected the palmitic acid C16:0 (14.27%) that is lower to that revealed in our result.

4.3 Bioactive Compounds characterization

4.3.1 Phenolic compounds

Phenolic compounds are responsible for physiological, biological, and biochemical functions, mainly because of their strong antioxidant activity, but also due to their properties as membrane stabilizers [60,61], they vary greatly among species [14-15]. The two flour samples allowed the identification of 10 phenolic compounds as shown in the Table 6.

Table 6. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, and identification of the phenolic compounds present in the acorn flours.

Peak	RT (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative Identification
1	4.32	270	169	125(100)	Gallic acid
2	5.12	270	613	451(34),301(100)	Dehydrated tergallagic acid-glucoside
3	7.06	280	783	481(9),301(33)	Pedunculagin (bis-HHDP-glucose)
4	7.98	271	951	907(100),783(16), 301(23)	Trigalloyl-HHDP-glucoside
5	13.18	361	463	301(100)	Ellagic acid hexoside
6	15.55	278	787	635(28),617(31),483(84),465(100),447(6), 423(73),313(10),271(9),169(5	Tetragalloyl-glucose
7	19.11	362	301	135(100)	Ellagic acid
8	12.51	283	635	465(21),301(31),169(100)	Trigalloyl-glucose
9	17.22	361	433	301(100)	Ellagic acid pentoside
10	18.51	359	447	301(100)	Methylellagic acid-pentose

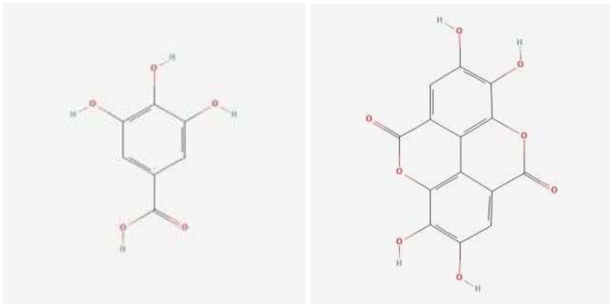
The tentative identification of each peak was carried out considering its chromatographic characteristics, namely, the mass to charge ratio (m/z), MS^2 fragmentation, retention time (RT, min), and maximum absorbance (λmax, nm), these data were compared to data obtained in the literature and with spectrograms of commercial standard compounds. The identified phenolic compounds were predominantly consisting of hydrolysable tannins, such as gallotannins, ellagitannins, and their derivatives. Five compounds were characterized in the class of hydrolysable tannins and another five as phenolic acids.

Regarding the phenolic acid, peak 1 ($[M-H]^-$ at m/z 169) and 7 ($[M-H]^-$ at m/z 301) was identified as gallic acid and ellagic acid respectively, according to standard compounds used in the quantification. Peaks 5 ($[M-H]^-$ at m/z 463), 9 ($[M-H]^-$ at m/z 433), and 10 ($[M-H]^-$ at m/z 447) were identified as ellagic acid derivatives. Mass spectrometric analysis revealed a molecular anion of ellagic acid hexoside at m/z 463. The MS^2 spectrum of this compound yielded an ion at m/z 301 (M-162), indicating the loss of a glucosyl unit. The peak at m/z 433 was identified as ellagic acid pentoside (M-132), corresponding to the loss of a pentosyl unit. Another peak at m/z 447 was identified as methyl ellagic acid pentoside (M-132-14), indicating the loss of a pentosyl and a methyl unit. The MS^2 spectrum of the m/z 301 ion and its maximum absorbance at 360 nm match the fragmentation pattern of ellagic acid.

Concerning hydrolysable tannins, peak 2 ($[MH]^-$ at m/z 613) was tentatively identified as dehydrated tergallagic acid-glucoside based on its spectrophotometric and spectrometric data, and previous data reported for acorns (*Quercus* spp.) and cork from *Quercus suber* L. [62,63]. This compound could derive from tergallagic acid C1-glucoside (MW 631) via intramolecular esterification with the loss of one molecule of water (18u). Peaks 3 ($[M-H]^-$ at m/z 783) and 4 ($[M-H]^-$ at m/z 951), showed the loss of one or more HHDP (hexahydroxydiphenoyl) residues (M-H302). Thus, the compound with a m/z 783, which differs by two mass units from the subsequent compound, was tentatively assigned as diHHDP-glucose and may result from the coupling of two adjacent galloyl groups in HHDP-digalloyl-glucose by intramolecular oxidation. The compound with m/z 951 was designated to a HHDP-trigalloyl-glucose. The MS^2 spectrum of this compound yielded a base peak at m/z 907 (M-44) indicating the loss of a carboxyl group, while the fragment ion at m/z 783 indicates the presence of an additional galloyl residue (M-H-152), probably linked to one HHDP residue (M-H-302) molecule by a C-C- bond, according to Gordon et al. 2011 [64]. Peaks 6 ($[M-H]^-$ at m/z 787) and 8 ($[M-H]^-$ at m/z 635), according to their MS fragmentation, show the loss of two or more galloyl (M−H-152) and/or gallate

(M−H170) units linked to a sugar, being identified as tetra-, and trigalloyl-hexoside, respectively.

Figure 9 shows the structure of gallic acid (1), the main compounds found in the acorn flour samples tested, as well as the structure of one ellagic acid (7), the second major compound found in both samples.



Gallic acid (peak 1)   Ellagic acid (peak 7)

Figure 9. Representation of gallic acid (1) and ellagic acid (7).

Table 7 shows the quantification of the phenolic compounds for both flours, the whole flour (SPM21) and the flour without tannins (SPM21F4). Overall, looking at the total phenolic compounds, there is a clear statistical difference between the amount of phenolics in the two samples, with the whole flour showing 37 mg/g of extract, while the flour without tannins only showing 9.6 mg/g extract. This reduction is due to the leeching process that removed tannins, which removed the phenolic compounds into the leeching solvent.

Table 7. Quantification of the phenolic compounds in the acorn four samples.

Quantification (mg/g of extract)			
Peak	Tentative Identification	SPM21	SPM21F4
1	Gallic acid	23±1*	5.5±0.2
2	Dehydrated tergallagic acid-glucoside	3.9±0.1	traces
3	Pedunculagin (bis-HHDP-glucose)	1.28±0.05	traces
4	Trigalloyl-HHDP-glucoside	nd	0.15±0.01
5	Ellagic acid hexoside	1.39±0.01*	1.267±0.003
6	Tetragalloyl-glucose	0.04±0.01	nd
7	Ellagic acid	2.13±0.03*	1.41±0.01
8	Trigalloyl-glucose	1.51±0.01	nd
9	Ellagic acid pentoside	1.66±0.02*	1.26±0.003
10	Methylellagic acid-pentose	1.45±0.01	traces

Total phenolic compounds	37±1*	9.6±0.2	

1.45±0.01 traces nd. – not detected. The quantification of the tentatively identified compounds was based on the calibration curves of authentic standards: gallic acid (y=131538x+292163, R2=0.9969, LOD = 8.05μg/mL and LOQ = 24.41μg/mL) for compounds 1 – 3 and 5 – 10; ellagic acid (y = 26719x – 317255, R^2 = 0.9986; LOD = 0.41 μg/mL and LOQ = 1.22 μg/mL) for compounds 4 and 11. An asterisk (*) means a significant difference between each sample with a significance of 0.05.

In Figure 10 it is possible to observe the HPLC chromatograms of the phenolic extract for both flours with the peaks corresponding to the identification observed in Table 6.

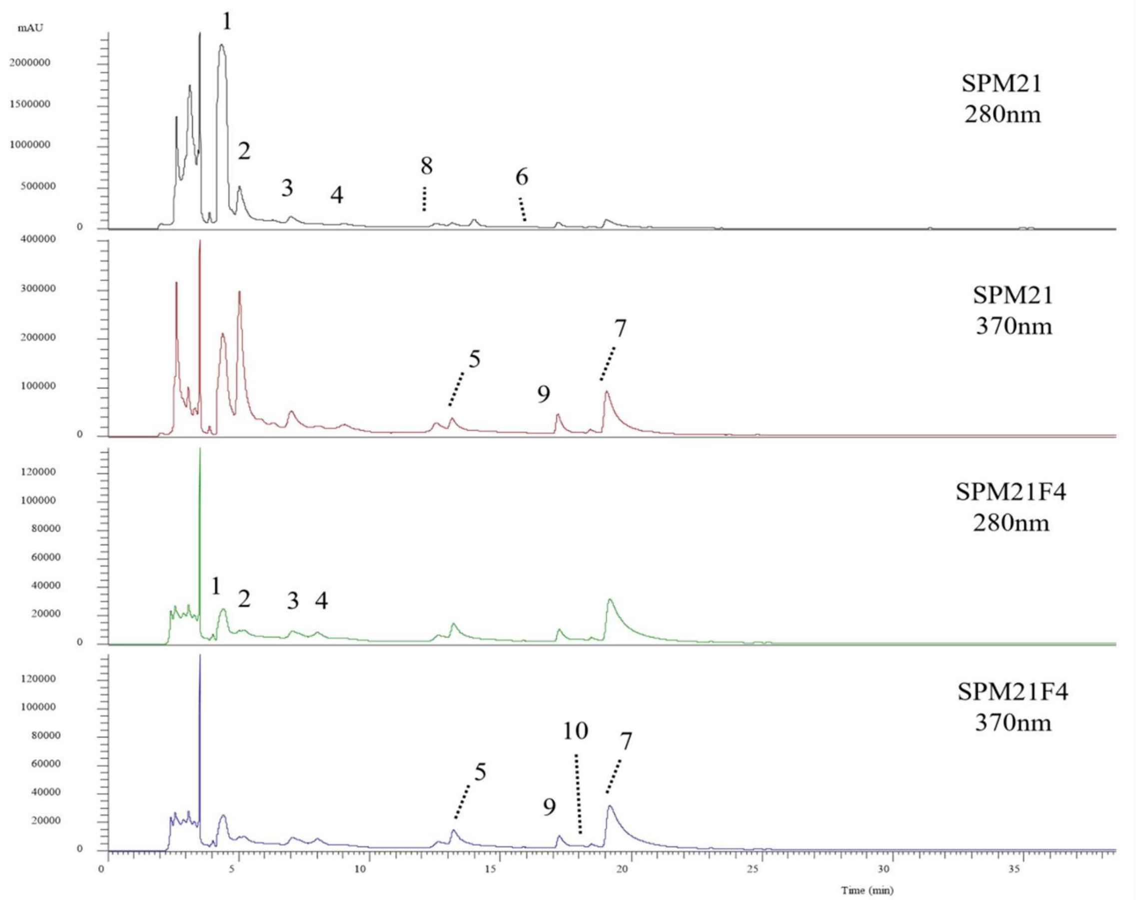


Figure 10. HPLC chromatogram of the phenolic extract for both flours, the full flour (SPM21) and the flour without tannins (SPM21F4), recorded at 280 nm and 370 nm.

Individually, the most abundant individual compound was gallic acid in the SPM21 flour, which reduced from 23 to 5.5 mg/g between the two samples. Peak 2 and 3, namely tergallagic acid-glucoside and pedunculagin (bis-HHDP-glucose) showed statistically significant reduction from SPM21 to SPM21F4. Leaching is used to eliminate tannins, bitter phenols, and other unpleasant compounds, which explains the reduction in these polyphenols [65]. This aligns with existing research showing a decrease in phenolic compounds and other phytochemicals in natural products after soaking [26,65]. All other peaks were completely removed with the leeching process, thus only being present in the SPM21 flour. The SPM21F4 derived from leached acorn nuts had a lower phenol content. Overall, the tendency was for a reduction of the phenolics and their quantities in the samples without tannins, most likely due to the leeching process, which used water and could have solubilized them and promoted their removal from the flour.

The results described in this thesis are in line with results reported by other authors (Cantos et al. [62], Ito et al. [66] and Fernandes et al. [63]). In the latter study, fractions of *Q. suber* samples were analyzed using HPLC and LC-DAD /ESI-MS. This analysis revealed the presence of various phenolic compounds, including low molecular weight phenolic acids, gallic acid, ellagic acid and their derivatives, and hydrolyzable tannins. However, this study mainly analyzed phenolic compounds present in the cork of *Q. suber* species and not in the acorn pulp itself.

As the species studied in this thesis was *Q. suber*, its added value is cork, meaning that not many records were found on the phenolic composition of acorn pulp or about the effects of leaching on the phenolic composition of these samples. That said, this not only makes this study innovative, but also makes it a more relevant contribution to the scientific community.

5. Conclusion

Nut flour is a hot topic in food research and has gained traction due to being a substitute for cereal flour in baked goods. Still, tannins which are astringent molecules are usually removed from these flours to improve taste and palatability of the final product. Still, the removal of tannins usually eliminates other nutrients and bioactive molecules. Thus, this work aimed at the comparing full acorn flour (SPM21) and the flour after tannin removal (SPM21F4) from *Q. suber*, through the nutritional evaluation and phenolic compounds characterization, to understand the grade of molecular removal.

As a result of the leaching procedure (in order to remove tannin) led to an increase in moisture, crude fat, starch, total carbohydrates and energy content in the flour SPM21F4. The soluble sugars (fructose, glucose and sucrose) were found exclusively in SPM21 flour, indicating that the treatment applied to the SPM21F4 sample affected its sugar content. This is likely because the sugars, being soluble, were drawn out of the flour during the leaching process, and thus were not present in the SPM21F4 sample.

The fatty acid profile showed significant variations; SPM21F4 contained higher levels of polyunsaturated (PUFA) and monounsaturated fatty acids (MUFA), whereas SPM21 had higher levels of saturated fatty acids (SFA). The leaching process primarily reduced MUFA and SFA levels, resulting in a relative increase in PUFA content in the tannin-free flour.

Regarding phenolic compounds analysis, SPM21 exhibited a higher phenolic content, which was expected, identifying ten distinct phenolic compounds. It is notable that, despite the discrepant values, the majority compound in both samples was gallic acid, followed by ellagic acid and its derivatives ellagic acid hexoside and pentoside. Overall, there is a statistically significant difference between the two samples.

amounts of phenolic compounds; the SPM21 flour had 36 mg/g of extract, while the SPM21F4 flour only showed 9.6 mg/g of extract. This decrease can be attributed to the removal of tannins by the leeching process, which also eliminated phenolic compounds into the leeching solvent. As *Q. suber*, the species examined in this thesis, is mainly valued for its cork, there is a scarcity of data on the impact of leaching on its phenolic composition. Therefore, this study stands out as both innovative and has a significant contribution to the scientific community.

The results of this study emphasize the feasibility of using tannin-free acorn flour as a nutritious and functional ingredient in the bakery industry since it can provide a valuable alternative to traditional flour offering enhanced nutritional benefits and appealing. sensory characteristics.

The future perspectives of this work are to quantify tocopherols (vitamin E), as they are important bioactive molecules, and it is expected to be found in high concentrations in acorns. Moreover, the bioactive properties of both flours are also very important to be evaluated, such as lipid peroxidation inhibition capacity (TBARS), cytotoxic activity and anti-microbial capacity.

Finally, production of bread with acorn flour with and without tannins will also be performed, followed by consumer acceptance studies.

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