



Article Phytochemical and Nutritional Profile Composition in Fruits of Different Sweet Chestnut (*Castanea sativa* Mill.) Cultivars Grown in Romania

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Abstract: Chestnut (*Castanea sativa* Mill.), a valuable fruit crop, is cultivated in small areas in Romania, mostly in the west, where the moderate continental climate has a slight Mediterranean influence. This work aims to investigate the bioactive characteristics (total polyphenols, total flavonoids and antioxidant activity), individual polyphenolic composition, phytochemical and nutritional HRMS screening profiles, sugar and mineral composition of six sweet chestnut cultivars, namely 'Marsol', 'Maraval', 'Bournette', 'Précoce Migoule' and 'Marissard' grown at Fruit Growing Research—Extension Station (SCDP) Vâlcea, in Northern Oltenia, Romania. Fruit samples were collected in two consecutive years, in order to study the impact of genetic variability between cultivars and the influence of the different climatic conditions corresponding to different cultivation years. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) allow the discrimination between the sweet chestnut fruits harvested in different years and different sweet chestnut cultivars. Analytical investigations revealed that sweet chestnut cultivars grown in Romania show similar bioactive, phytochemical and nutritional composition to cultivars grown in the large European chestnut-producing countries, indicating the high adaptation potential of the chestnut in the temperate continental zone with small Mediterranean influences characteristic of the southwestern area of Romania.

Keywords: sweet chestnut; bioactive characteristics; phenolic compounds; HRMS fingerprints; sugar profile; mineral composition; statistical analysis

1. Introduction

The sweet chestnut is the fruit of the *Castanea sativa* Mill., which belongs to the genus *Castanea*, of the *Fagaceae* family, and is cultivated especially in Mediterranean Europe [1]. Sweet chestnut is an important resource in Europe due to its economic value associated with fruit, wood and tannin production and indirectly with honey production, but also due to its cultural value [2]. *Castanea sativa* Mill. is also commonly known as European chestnut and has a large distribution in Spain, France, Greece, Italy, Portugal, and Turkey [3].

Nutritionally, chestnuts have interesting characteristics, containing significant amounts of carbohydrate dietary fiber, but small amounts of crude protein (2–4%) and low levels of crude fat (predominated by unsaturated fatty acids) (2–5%) compared to typical walnuts (walnuts, almonds, hazelnuts) [4,5], thus being a good source of energy with multiple health-beneficial effects. Chestnuts are also low in fat, thus helping to decrease cholesterol levels and they contain a high amount of vitamin C, macro- (K, P, Mg, Ca, Na) and micro-nutrients (Mn, Fe, Zn and Cu) [6]. The fruits also have a significant antioxidant activity associated with polyphenolic and organic contents [7].



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Thus, chestnut fruits have become very important in the human diet due to their nutritional composition and health benefits, for example, their use in gluten-free diets in celiac disease [8], reducing abdominal adiposity [9] and reducing coronary heart disease and cancer rates [3]. The growing demand for traditional foods has converted chestnuts into a value-added resource with considerable potential as functional foods or food ingredients. The nuts are consumed in roasted or boiled form or for the development of different added value products in the cake and candy industry [10]. Considering the fact that cooked chestnuts are a good source of phenolics (gallic and ellagic acids) and organic acids (citric acid) and have low fat contents [7], properties that are associated with positive health benefits, the development of new products based on chestnuts should be encouraged [11]. Therefore, over the last few decades, the chestnut industry has significantly grown in Europe, especially in the production of marron glacé, purées and chestnut flour, which find increasing application as an ingredient in gluten-free diets [12], such as the production of pasta by incorporating chestnut flour and bee pollen [13]. In addition, chestnut extracts can be used in the food industry as functional ingredients and natural preservatives aiming to replace the synthetic ones capable of improving the shelf-life and nutritional value of products [14,15]. Furthermore, chestnut shells as the main byproduct generated from chestnut processing are currently used as fuels [16], for the production of lignin biopolymer and bioethanol following a biorefinery approach [17], but also can be a source of hydrolyzable tannins as natural pigments for food and pharmaceutical industries [18], as a bioactive ingredient for nutraceutical and cosmetic industries [14]. Different marketed food supplements (sold as tablets or capsules) and herbal medicinal products (powders, extracts, essential oil) based on chestnuts have been obtained over time in order to protect the health of the population.

Therefore, due to the increased economic interest in the use of sweet chestnuts in the food industry, there was a need to develop selected cultivars with enormous potential on human health associated with the consumption of chestnuts and processed products based on chestnuts [19]. Additionally, in order to increase chestnut production and resistance to chestnut-specific diseases, some hybrids have appeared over time [11].

The nutritional composition and bioactivity of fresh sweet chestnuts show differences between cultivars [2,20], producing regions, harvesting year [21], soil and climatic conditions (temperature, sun exposure and precipitation) [22], but also cultivation techniques, for example, nutrients, minerals, irrigation and diseases and pests [23,24].

The increased market demand and consumer awareness imposes the development of reliable methods able to distinguish between different cultivars, highlighting high quality products in terms of sensorial and qualitative properties and high bioactive composition. In the last few decades, different methodologies have been used to characterize and distinguish between different sweet chestnut cultivars, including morphological characteristics [25] and chemical composition addressing the proximate analysis including dry mass, ash quantity, total fat, total protein [7,26], total carbohydrates, total sugar, invert sugar, starch, sucrose [3], but also mineral contents (Ca, Mg, Fe, Mn, Cu, Zn, P, Na and K) [3,4,27], total polyphenols (mg GAE/g) and antioxidant activity (µmol Trolox equivalent/g) dry weight basis [4,5] and organic acids (oxalic, cis-aconitic, citric, ascorbic, malic, quinic and fumaric acids) [7,28], free amino acids [26], sugars profile [29,30].

For decades, conventional extraction methods including maceration or Soxhlet extraction using different polar solvents (methanol, ethanol, chloroform and petroleum ether) were the most used to extract bioactive compounds from a natural matrix, and which, due to environmental, economic and safety concerns, presents a huge limitation for an industrial application. Given these disadvantages, more sustainable extraction methods, including ultrasonic extraction, microwave extraction, supercritical fluid extraction and enzymatic extraction were promoted for the extraction of polar bioactive compounds from natural sources [18,31].

Chestnut growing areas in Romania cover a total area of 3160 ha distributed on a discontinuous area, consisting of long bands situated on the foothills of the Carpathians,

mostly in the west part of Romania, where the moderate-continental climate has a slight Mediterranean influence. Chestnut natural distribution cover two principal centers, namely Maramureş (the hilly foothills of Baia Mare) and Oltenia (subcarpathian hills of Oltenia on the territory of Gorj, Mehedinți and Vâlcea counties) and other several small areas on Southeast of the Oriental Carpathians and Northwest and Southwest of Transylvanian plateau [32]. The semi-spontaneous flora of Northern Oltenia contains many biotypes of sweet chestnut (Castanea sativa Mill). Since 1998, several French cultivars, which are hybrids between Japanese chestnut (Castanea crenata Siebold & Zucc.) and European chestnut (Castanea sativa Mill.) were introduced at the Fruit Growing Research—Extension Station (SCDP) Vâlcea for testing and they have proved to yield well in the given conditions. Physical characteristics (diameters, height, shape index and size index, mass, volume and specific weight), nutritional composition (water (%), titrable acidity (g malic acid/100 g), lipids (%), proteins (%) and bioactive characteristics (total polyphenols, total flavonoids, antioxidant activity and some individual polyphenolic compounds) of these cultivars were previously addressed [33] and supplementary characteristics are required in order to make a detailed characterization and comparison of these six chestnut cultivars, in order to provide valuable information for selection of the chestnut cultivar with high quality bioactive characteristics that can be cultivated for the development of different value-added food products with multiple benefits on human health.

Therefore, this work aims to strengthen our knowledge about the chemical and nutritional composition of the six sweet chestnut cultivars of French origin, namely 'Marsol', 'Maraval', 'Bournette', 'Précoce Migoule' and 'Marissard' in order to find distinctive features useful for authenticating a certain chestnut cultivar, providing an important economic advantage. For that, the bioactive characteristics (total polyphenols, total flavonoids and antioxidant activity) examined by UV-Vis spectrophotometric methods, UHPLC-MS/MS phenolic compound profile (phenolic acids, flavonoids), together with a non-target UHPLC-MS/MS screening profile, the sugar profile (sucrose, fructose, glucose, maltose) by HPLC-ELSD and elemental composition (Ca, Mg, Na, K, Fe, Pb, Ni, Cu, Cr) by F-AAS were determined in the studied chestnut cultivars in order to complete the information about the nutritional and bioactive composition of all these cultivars. In addition, we studied the influence of the harvest year on the bioactive characteristics and the content of the specific bioactive compounds. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were used in order to discriminate between the different sweet cultivars grown in Romania.

2. Materials and Methods

2.1. Plant Material and Sampling Site

Fruit samples were collected from a field trial planted in 1998 at Fruit Growing Research-Extension Station (SCDP) Vâlcea, located in Bujoreni (45°08'26.55" N; 24°22'01.02" E), near Râmnicu Vâlcea, in the Southwest of Romania. SCDP Vâlcea belongs since 2010 to the University of Craiova. The trial contains French sweet chestnut cultivars as: 'Précoce Migoule CA 48', 'Bournette CA 112', 'Marsol CA 07', 'Marissard CA 122', 'Marigoule CA 15' and 'Maraval CA 74', all of them provided by INRA-UREFV Bordeaux, France. Trees are grafted on Castanea sativa seedling rootstock and were planted in the trial orchard in 1998 and were grown under the same pedoclimatic conditions and agronomic management practices at SCDP Valcea, Northern Oltenia, Romania. The orchard soil was alluvial, clayey-loam type, slightly acid (pH = 5.2), the mineral content level was good, and organic matter level was poor. The climate is fairly mild, and annual rainfall is about 700-800 mm. Chestnut fruits were harvested at maturity, at the end of September through the middle of October, during two consecutive years (2016 and 2017). They were collected randomly from different trees, for each cultivar 1 kg of samples were collected and within a week from harvest, the nuts were hand peeled and then dehydrated at 40 °C using a Biovita drier (Globus Transport, Cluj Napoca, Romania), ground to fine powder by using a Retsch

200 mill (Verder Scientific, Haan, Germany) and then lyophilized (Labconco freeze dryer) (Labconco Corporation, Kansas, MO, USA) for further analysis.

2.2. Standards and Reagents

All solvents were of high-performance liquid chromatography (HPLC) grade purchased from Merck (Darmstadt, Germany) (methanol, acetonitrile), Folin–Ciocalteu phenol reagent (2 N), radical scavenging assay reagents DPPH and 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylicacid (Trolox) were purchased from Sigma-Aldrich (St Louis, MO, USA) and ultra-pure water was produced by a Milli-Q Millipore system (Bedford, MA, USA). Other reagents (formic acid, nitric acid, hydrogen peroxide) were analytical grade and purchased from Merck (Darmstadt, Germany). Phenolic standards (gallic, syringic, p-coumaric, caffeic, chlorogenic, ferulic, p-hydroxybenzoic, 3,4-dihydroxybenzoic and trans-cinnamic acids; (+)-catechin and (–)-epicatechin, rutin, quercetin, naringin, hesperitin, myricetin, apigenin, galangin, kaempferol, isorhamnetin, chrysin, pinocembrin, pinostrobin and resveratrol) and sugar standards (glucose, fructose, sucrose and maltose) were purchased from Sigma-Aldrich (Steinheim, Germany). High purity multielement calibration standard solution ICP Multi-Element Standard Solution IV CertiPUR (1000 mg/L of each element in HNO3 65%), and ICP Multi-Element Standard Solution XXI CertiPUR (10 mg/L of each element in HNO3 65%) were purchased from Merck KGaA Frankfurter, Darmstadt, Germany.

2.3. Extraction Protocols

Polyphenolic compounds were extracted from freeze-dried subsamples (0.25 g) using a 25 mL mixture of methanol: water (70:30, v/v), under the action of ultrasound, at a temperature of 60 °C, for 30 min. The resulted suspension was centrifuged at 10,000 rpm for 15 min and the supernatant was concentrated at 40 °C under reduced pressure using a TurboVap MP6 (Buchi, Switzerland) and then diluted with methanol to a final volume of 3 mL. The resulting extracts were filtered through 0.45 µm PTFE (polytetrafluoroethylene) membrane syringe filters (Merck Millipore, Darmstadt, Germany) before the UV-Vis spectrophotometric and UHPLC-MS/MS determinations.

Sugars were extracted from a 2.5 g sample using 10 mL ultrapure water, under ultrasounds, at 60 °C, for 30 min. The resulting suspension was centrifuged at 10,000 rpm for 10 min, followed by the quantitative recovery of the analytes by two washing steps. The combined extracts were diluted with ultrapure water to a final volume of 20 mL, followed by filtration through 0.45 μ m PTFE membrane syringe filters before HPLC-ELSD determination.

In order to estimate the elemental content, chestnut samples were extracted by microwave acid digestion using a TOP microwave sample preparation system (Analytik Jena, Jena, Germany) equipped with closed Teflon vessels. In brief, 0.4 g of sample was placed in the Teflon digestion vessel previously cleaned with 10% nitric acid, followed by the addition of 6 mL of concentrated nitric acid 65% (spectroscopic grade, Merck, Darmstadt, Germany) and 1 mL of hydrogen peroxide 30% were added. The vessels were closed and placed in the rotor, followed by the application of the digestion program composed by three step temperature programs, namely: (i) pressure 40 bar, ramp 5 min, temperature 170 °C, time 10 min; (ii) pressure 40 bar, ramp 1 min, temperature 200 °C, time 15 min; and (iii) cooling. After the digestion process, digested samples were quantitatively transferred with ultra-pure water into a 50 mL volumetric flask, filtered and then subjected to F-AAS determination.

2.4. Analytical Determinations

2.4.1. Determination of Total Phenolic (TP) and Total Flavonoid (TF) Contents and Antioxidant Activity (AA) by UV-Vis Spectrophotometric Determinations

Colorimetric spectrophotometric determinations of the chestnut extracts (total polyphenols—TP, total flavonoids—TF and antioxidant capacity—AC) were performed using a Specord 250 Plus UV-Vis spectrophotometer (Analytic Jena, Germany) equipped with 1 cm path length quartz cells. The total polyphenol content (TP) was evaluated following the Folin–Ciocalteu colorimetric method as presented by Ciucure et al. [34], using gallic acid as a reference substance, while total flavonoids content (TF) was determined by the aluminum chloride method as previously presented [35], using rutin as reference substance. Antioxidant capacity in the chestnut fruits was assessed by the DPPH method, using Trolox as a reference substance. All the experiments were performed in duplicate, and results were expressed as mean of gallic acid equivalents (mg GAE/g lyophilized chestnut), mean of rutin equivalents (mg RE/g lyophilized chestnut) and μ mol/L Trolox equivalents/g lyophilized chestnut), respectively.

2.4.2. Phenolic Compounds Profile by UHPLC-DAD-ESI/MS

All experiments were performed using an UltiMate 3000 UHPLC system (ThermoFisher Scientific, Bremen, Germany), coupled to a Q ExactiveTMFocus Hybrid Quadrupole-OrbiTrap mass spectrometer) equipped with heated electrospray ionization (HESI) probe (ThermoFisher Scientific). Two chromatographic columns: Accuacore PFP (50 mm × 2.1 mm, 2.6 μ m) and Accuacore PFP (100 mm × 2.1 mm, 2.6 μ m) from Thermo Fisher Scientific were used for the separation of the compounds, under the gradient elution of a binary solvent system consisting of solvent A (water with 0.1% formic acid, v/v) and solvent B (methanol with 0.1% formic acid), at a flow rate of 0.3 mL/min. The mass spectrometer was operated in negative mode, in a range between 75–1000 m/z, at a resolution of 70,000. The UHPLC gradient for mass screening and the operation conditions of the mass spectrometer were previously optimized [34]. Calibration solutions consisting of a mixture of caffeine, methionine-arginine phenylalanine-alanine-acetate (MRFA), sodium dodecyl sulfate, sodium taurocholate, and Ultramark 1621 were used for the calibration of the Orbitrap mass analyzer.

For phenolic compound quantification, calibration was performed in the concentration range between 0 and 10 mg/L for each of the phenolic acids and flavonoids by serial dilution with methanol of the stock standard mixture of 100 mg/L [34]. Analytical performances of the target HRMS analysis of polyphenolic compounds from chestnuts were expressed in terms of linear range, correlation coefficients (R²), limit of detection (LOD), and limit of quantification (LOQ), and recovery (by spiked experiments 0.5 mg/L level). The limit of detection (LOD) and the limit of quantification (LOQ) for each target standard compound were determined by the diluted standard solution until the signal-to-noise ratios (S/Ns) were 3:1 and 10:1, respectively. The results were expressed as mg/g of lyophilized chestnut sample.

The data were purchased and processed using the Xcalibur software package (Version 4.1). The Total Ion Current (TIC) profile results were found by monitoring the intensity of all the ions produced and acquired in every scan during the chromatographic run. For structural information, data-dependent experiments were performed by acquiring MS/MS spectra of the most intense ions resulting during the HRMS scan event. Normalized collision energy at 30%, a minimum signal threshold at 200, and an isolation width at 3.0 were used. ChemSpider reference spectral database (www.chemspider.com (accessed on 7 February 2022)) was used as a reference library to identify other representative bioactive compounds from the chestnut extracts. For the non-target UHPLC-MS/MS screening, data processing, analysis and interpretation were performed with Compound Discoverer v. 2.1 (Thermo Scientific, San Jose, CA, USA) software using an untargeted metabolomics working template.

2.4.3. Sugars Profile by HPLC-ELSD

Chestnut sugar composition in terms of glucose, fructose, sucrose and maltose was determined using an HPLC Surveyor Plus system (Thermo Fischer Scientific Inc., San Jose, CA, USA) using an evaporative light scattering detector (Alltech 3300 ELSD, Grace Davison Discovery Sciences) operated by Chrome Quest software. The chromatographic separation of sugars was achieved using an APS-2-Hypersil amine bonded phase column (250 mm × 4.6 mm, 5 μ m) and isocratic elution of acetonitrile/water (85:15, v/v) mobile phase at 0.8 mL/min flow rate. Chromatographic and ELSD detector parameters were set as described in [36]. The amounts of glucose (G), fructose (F), sucrose (S) and maltose (M) in chestnuts were quantified using external calibration curves and the results were expressed as mg/g of lyophilized sample.

2.4.4. Elemental Content Determination

The determination of the amount of Ca, Mg, Na, K, Fe, Pb, Ni, Cu, Cr in sweet chestnuts fruits of different cultivars was carried out by flame atomic absorption spectroscopy (F-AAS) NOVAA 300 model with an air/acetylene flame. Hallow cathode lamps of the different metals were used as the radiation sources. The analytical measurements were based on time-averaged absorbance. Instrumental parameters were previously presented [37] The digested samples were directly injected into the spectrophotometer. External calibration curves were set up for each of the elements using calibration solutions prepared by successive dilution of standard solutions. All the obtained calibration curves showed very good correlation coefficients ($\mathbb{R}^2 > 0.995$) and standard deviation values <10%. All determinations were performed in duplicates.

2.5. Data Analysis

All the analyses were made in duplicate. Statistical differences between different cultivars and harvest years were tested using one way ANOVA followed by Duncan's new multiple range test with a 0.05 significance level. For the ANOVA analysis, the factors taken into consideration in this study were the cultivar and harvest year and the interaction between these factors. A principal component analysis (PCA) and Hierarchical Cluster Analysis were carried out on the data matrix including 20 rows (two repetitions for 10 samples) and 22 phenolic variables resulting from the UHPLC-MS analyses. ANOVA and PCA (including Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy) were performed with statistical software packages Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and XLSTAT v. 15.5.03.3707 (Addinsoft, Paris, France).

3. Results

It is known that the climatic conditions (e.g., temperature and precipitations), cultivars, geographical area, soil nutrients and water availability may affect the bioactive composition of chestnut fruits [18]. Considering that the samples from this study were obtained in the same climatic and pedological conditions, the differences in the bioactive characteristics, individual phenolic compounds, phytochemical and nutritional profiles, sugar composition and macro- and micro-nutrients can be attributed due to the genetic differences between the studied cultivars or to the differences between the harvest years.

3.1. Evaluation of Total Polyphenolics (TP), Total Flavonoids (TF) and Antioxidant Activity (AA) of Chestnut Cultivars

Mean TP, TF and AA values for the studied chestnut cultivars during two consecutive years, 2016 and 2017, investigated in this study and for 2015 reported in a previous study [33] are presented in Figure 1.

High contents of TP correspond to 'Marissard', with average values of 12.69 mg GAE equivalent/g DW, followed by and 'Précoce Migoule', 'Marigoule' and 'Maraval' cultivars with average values of 6.11, 4.84 and 4.10 11 mg GAE equivalent/g DW, respectively. The obtained values were in agreement with data from other studies [5]. Average values of TF ranged between 1.57 mg rutin equivalent/g DW in 'Marsol' and 8.64 mg rutin equivalent/g DW in 'Marissard'. Differences between antioxidant activity values, expressed as µmoli Trolox equivalents/g were observed among the cultivars, with a trend similar to the one observed for TP levels. Good correlation coefficients were obtained between Total Polyphenolics with antioxidant activity ($R^2 = 0.9226$) (Figure S1), suggesting that phenolic compounds were the main antioxidant components of chestnuts. However, determining the contribution of each class of bioactive compounds to total antioxidant activity may be difficult due to the synergistic effect and interaction between different substances [38].





Figure 1. Antioxidant activity (AA) expressed as µmoli Trolox equivalents/g, Total polyphenols (TP) expressed as mg GAE equivalents/g and Total Flavonoids (TF) expressed as mg rutin equivalents/g of the six chestnut cultivars for three consecutive years.

According to the Duncan test at $p \le 0.05$, based on AA, TP and TF, no significant differences were observed between the studied cultivars, only based on AA and TP variables, the 'Marissard', 'Bournette' and 'Marsol' cultivars shows some differences compared to the other cultivars (Figure S2).

For each studied cultivar, variations in TP, TF and AA were observed depending on the harvest year, this being correlated with the climatic conditions specific to each harvest year. It is well known that polyphenols represent important plant metabolites with physiological roles for plant life, which accumulate in plants as a response to stressful conditions, such as harmful environmental conditions (drought, extreme temperatures, pollution, ultraviolet radiations) [39]. Therefore, climatic conditions in the 2015 harvest year were more favorable for obtaining harvest reach in polyphenols, being warmer and drier, compared to 2016 and 2017 (Table 1), the higher TP, TF and AA being obtained for chestnuts fruits harvested in 2015.

	Tem	perature at 2 m He	eight		Polativo		
Year	Mean Temperature T _{avr,} (°C)	MeanMinimumMaximumuperatureTemperatureTemperaturevvr, (°C)T _{min} , (°C)T _{max} , (°C)		Rainfall (mm)	Humidity (%) at a Height of 2 m	Number of Days with Rainfall	
2015	12.6	-16.1	37.3	706	70	126	
2016	11.9	-14.0	35.2	746	73	135	
2017	12.0	-19.1	38.6	802	72	136	

Table 1. Climatic data for Râmnicu Vâlcea area during study period (source of data: www.rp5.ru (accessed on 7 February 2022)).

It is important to note that, in order to obtain crops with high phytochemical and nutritional potential, it is necessary for the fruit to reach maturity. Thus, in order to determine the amount of heat or the thermal time necessary to reach the beginning and the end of sweet chestnut harvesting the Growing Degree Hours (GDH) and Growing Degree Days (GDD) were estimated. GDH were calculated from December 1st till start and end of harvesting time the next year, while GDD were calculated from May 1st till the start and end of harvesting time as average hours and, respectively, daily temperatures above 6 °C (base temperature) and below 30 °C (highest optimum temperature before stress) [40,41]. The obtained average values (during 2015, 2016 and 2017 harvest years) ranged from 61,832 GDH for 'Précoce Migoule' to 63,670 GDH for 'Maraval', while average values for GDD ranged from 2218 °D for 'Précoce Migoule' to 2295–2218 °D for 'Maraval' (Table S1). Our results are consistent with literature data which indicate 60,000 GDH accumulation for the chestnuts located in cooler regions [42] and values between 1900–2400 °D between May to October for chestnut regions [41].

3.2. Target UHPLC-MS/MS Analysis of Phenolic Compounds Biomarkers in the Investigated Chestnut Cultivars

Target UHPLC-MS/MS method was applied for the quantitative determination of 27 phenolic compounds in the methanolic extracts obtained from fruits of different sweet chestnut cultivars. The peak identification was performed by comparing retention times (t_R) and mass spectra (accurate mass and mass fragments) with those of reference standards (Table S2). Quality performances of the UHPLC-MS/MS method evaluated in terms of linearity, correlation coefficients (R^2), LODs, LOQs and recovery were detailed in Table S2. The R^2 coefficient for all standards was higher than 0.95, showing good linearity. The values of LODs and LOQs were in the range of 0.12–1.2 µg/g DW and 0.4–4.0 µg/g DW, respectively. The recovery rates of all the components, performed by spiked experiments, ranged from 80% to 101%. The results demonstrated that the developed UHPLC-MS/MS method was sensitive and accurate for the quantitative determination of all the tested constituents in chestnut fruits. The analyses were conducted in duplicate for each chestnut sample and the data were expressed as mean values and standard deviations for each cultivar harvested in both, 2016 and 2017. The result of the quantification of the target compounds in chestnut extracts were shown in Table 2.

Table 2. The results of the quantitative analysis of phenolic compounds (average values \pm standard deviation) in fruits of different sweet chestnut cultivars harvested in 2016 and 2017 (mg/g DW).

Phenolic Compounds	'Marigoule' (MG)	'Précoce Migoule' (PM)	'Bournette' (B)	'Marsol' (MS)	'Maraval' (MV)	'Marissard' (MRS)
Gallic acid	1.035 ± 0.466	0.659 ± 0.156	1.670 ± 0.574	0.508 ± 0.118	0.158 ± 0.005	1.282 ± 0.006
3,4-Dihydroxybenzoic acid	0.068 ± 0.088	0.140 ± 0.187	NF	NF	NF	0.009 ± 0.001
4-Hydroxybenzoic acid	0.052 ± 0.051	0.200 ± 0.262	0.016 ± 0.018	0.026 ± 0.025	0.003 ± 0.001	0.008 ± 0.001
Chlorogenic acid	0.001 ± 0.0004	0.001 ± 0.001	NF	0.002 ± 0.001	NF	0.001 ± 0.0009
Syringic acid	0.007 ± 0.008	0.024 ± 0.012	0.008 ± 0.010	0.002 ± 0.003	NF	0.010 ± 0.001
Caffeic acid	0.010 ± 0.000	0.007 ± 0.010	0.011 ± 0.001	0.006 ± 0.008	0.009 ± 0.001	0.011 ± 0.001
p-Coumaric acid	0.017 ± 0.002	0.030 ± 0.020	0.023 ± 0.005	0.015 ± 0.009	0.009 ± 0.002	0.023 ± 0.002
t-Ferulic acid	0.018 ± 0.006	0.015 ± 0.006	0.022 ± 0.020	0.024 ± 0.017	0.009 ± 0.001	0.023 ± 0.001
Ellagic acid	0.757 ± 0.600	0.349 ± 0.143	6.211 ± 6.807	0.217 ± 0.242	0.056 ± 0.006	2.902 ± 0.037
Cinnamic acid	0.012 ± 0.000	0.019 ± 0.001	0.018 ± 0.011	0.023 ± 0.003	0.002 ± 0.001	0.029 ± 0.002
(+)-Catechin	0.332 ± 0.304	0.258 ± 0.294	0.090 ± 0.082	0.036 ± 0.051	0.174 ± 0.007	0.363 ± 0.013
(–)-Epicatechin	0.005 ± 0.004	0.007 ± 0.008	0.002 ± 0.002	0.003 ± 0.003	0.001 ± 0.001	0.010 ± 0.001
Quercetin	0.006 ± 0.004	0.005 ± 0.002	0.001 ± 0.0002	0.001 ± 0.001	NF	0.004 ± 0.001
Myricetin	0.044 ± 0.001	0.044 ± 0.010	0.042 ± 0.002	0.038 ± 0.002	0.031 ± 0.008	0.045 ± 0.002
Naringin	0.006 ± 0.004	0.009 ± 0.012	0.006 ± 0.002	0.003 ± 0.001	NF	0.009 ± 0.001
Rutin	0.001 ± 0.0004	0.004 ± 0.001	0.004 ± 0.0002	0.001 ± 0.0003	NF	0.010 ± 0.001
Isorhamnetin	0.002 ± 0.002	0.001 ± 0.0007	0.001 ± 0.0001	NF	NF	0.001 ± 0.0010
Apigenin	NF	NF	NF	NF	NF	NF
Hesperidin	NF	NF	NF	NF	NF	NF
Kaempferol	NF	NF	NF	NF	NF	NF
Pinocembrin	0.002 ± 0.0002	<loq< td=""><td>0.002 ± 0.0002</td><td>0.002 ± 0.0002</td><td><loq< td=""><td><loq< td=""></loq<></td></loq<></td></loq<>	0.002 ± 0.0002	0.002 ± 0.0002	<loq< td=""><td><loq< td=""></loq<></td></loq<>	<loq< td=""></loq<>
Chrysin	0.001 ± 0.001	NF	NF	NF	NF	NF
Galangin	0.003 ± 0.003	<loq< td=""><td>NF</td><td>NF</td><td><loq< td=""><td>NF</td></loq<></td></loq<>	NF	NF	<loq< td=""><td>NF</td></loq<>	NF
t-Resveratrol	0.007 ± 0.010	0.002 ± 0.0002	0.001 ± 0.0003	0.001 ± 0.0008	NF	0.050 ± 0.056

NF-not found.

To evaluate the contribution of each class to the total polyphenolic composition, the phenolic bioactive compounds were grouped in the following classes: benzoic acids (gallic, protocatechuic, p-hydroxybenzoic and syringic acids), cinnamic acids (caffeic, p-coumaric, ferulic, cinnamic and chlorogenic acids), catechins ((+)-catechin and (-)-epicatechin) and flavanols (naringin, rutin, myricetin, quercetin, isorhamnetin, apigenin, pinocembrin) and t-resveratrol) (Figure 2). Apigenin, hesperidin and kaempferol were not identified in chestnut extracts, while chrysin and galangin were quantified in very low amounts only in 'Marigoule' or quantified below LOQs (Table 2).

Within the polyphenolic groups, differences were observed among chestnut cultivar and harvest year, higher amounts of polyphenolic compounds corresponding to 'Marissard', 'Bournette' and 'Précoce Migoule'.



Figure 2. Polyphenolic profile of the analyzed chestnut cultivars harvested in 2016 and 2017.

Benzoic acids and catechins, recognized for their antioxidant, anticancer, antiinflammatory and antimicrobial potential [43], represented the main component of the polyphenolic chestnut extract and among them, gallic and ellagic acids were quantified between 0.162–2.077 mg/g DW and 0.046–2.928 mg/g DW, while (+)-catechin was quantified between n.d. and 0.547 mg/g DW. p-Coumaric, ferulic and cinnamic acids were the representative for the cinnamic acids class, with values between 0.008–0.045 mg/g DW for p-coumaric acid, 0.008–0.036 mg/g DW for ferulic acid and 0.002–0.028 mg/g DW for cinnamic acid. Among flavanols, myricetin, naringin, rutin and quercetin were quantified in higher amounts, with values ranging between 0.036–0.051 mg/g DW for myricetin, n.d.—0.018 mg/g DW for naringin, n.d.—0.011 mg/g DW for rutin and n.d.—0.009 mg/g DW for quercetin. t-Resveratrol was quantified with values between 0.001 and 0.014 mg/g DW, with higher amounts in 'Marigoule' and 'Marissard' in the 2017 harvest year. The results of the quantitative analysis are similar to those obtained in other studies [2,5,11].

According to Duncan's test at $p \le 0.05$, based on individual phenolic compound profiles, no significant differences were observed between the studied cultivars, only some differences can be observed based on gallic and cinnamic acids and rutin variables (Supplementary Figure S3). Based on gallic acid content, 'Bournette' and 'Maraval' are grouped differently compared to the rest of the cultivars, with a high amount of gallic acid corresponding to 'Bournette', while lower quantities corresponded to 'Maraval'. According to cinnamic acid content, 'Marissard' and 'Marsol' were grouped separated toward 'Bournette', which shows lower cinnamic acid content (average value). 'Marissard' displayed the highest rutin content (0.011 mg/g DW), being grouped separately from the rest of the chestnut cultivars.

The most important pharmacological properties of gallic and cinnamic acids are attributed to their antioxidant and anti-inflammatory potentials, demonstrating a broad range of beneficial activities, such as antimicrobial, antioxidant, gastroprotective, neuroprotective, cardioprotective, anticancer, antidiabetic and anti-inflammatory [44,45], while rutin shows multiple health-promoting effects, such as neuroprotective, nephroprotective, hepatoprotective, cardioprotective, and retinoprotective activities, but also antibacterial and antifungal activities [46]. Therefore, chestnuts can be considered as valuable natural sources for these bioactive compounds, the phytochemical chestnut extract being suitable for introduction as dietary supplements [47].

For exploratory data analysis purposes, principal components analysis (PCA) and Hierarchical Cluster Analysis (HCA) were used as unsupervised statistical methods in order to discriminate between fruits of different chestnut cultivars, based on the individual phenolic compounds profile. The first two principal components (PC 1 and PC 2) with 54.47% of the whole variances were extracted from PCA analysis. The other main components that had a minor effect on the model were removed. The obtained low value for the whole variances can be attributed to the small number of samples investigated for each category. The distribution in the PC1–PC2 score plot plane of the different chestnut cultivars is sown in Figure 3.





No clear discrimination can be observed between the fruits of different chestnut cultivars. Along the PC1 axis most cultivars harvested in 2017 can be discriminated, except 'Maraval' and 'Marsol', while the PC2 axis seems to discriminate the 'Précoce Migoule' cultivar from the others. It can be noticed that phenolic compounds, such as gallic, ellagic, cinnamic, chlorogenic and caffeic acids, (+)-catechin and (–)-epicatechin, have the largest contribution to the total variation, along PC1, these compounds being quantified in high amounts in the 2017 harvest year, compared with the 2016 harvest year.

Hierarchical Cluster Analysis was used as an exploratory tool to assess the dissimilarities between the fruits of different chestnut cultivars (Figure 4). At the dissimilarity level of 7, class 1 (C1) refers to 'Précoce Migoule', 'Marsol' and 'Maraval' cultivars, being the cultivars with a lower content of phenolic compounds, compared with the others. Chestnut cultivars with high polyphenolic composition, namely 'Marissard', 'Bournette' and 'Marigoule' were grouped in C2 and C4 groups, while the 'Bournette' cultivar harvested in 2016 forms a distinct group, being the cultivar with lower catechins content.



Figure 4. Dendrogram of the fruits of different chestnut cultivars ('Précoce Migoule'—PM, 'Bournette'—B, 'Marsol'—MS, 'Marissard'—MRS, Marigoule—MG and 'Maraval'—MV): C1, C2, C3 and C4—classes grouped by dissimilarities criteria.

3.3. Untarget UHPLC-MS/MS Analysis of Biomarkers and Their Metabolite Profiles in Fruits of Different Chestnut Cultivars

Identification and quantification of polyphenols in fruits are of major importance because of their beneficial impact on human health due to their additive and synergistic effects on radical scavenging [48]. Thus, an analytical approach based on a non-target UHPLC-Q-Orbitrap HRMS method was carried out aiming to identify other bioactive compounds and specialized metabolites that occur in methanolic extracts of chestnut fruits, which are responsible for their antioxidant and anti-inflammatory activities, and to highlight the differences between extracts of different chestnut cultivars. Data processing analysis used Compound Discoverer software using an untargeted metabolomics working template, which comprises an untargeted workflow which includes options for peak picking performs RT alignment, prediction of the molecular formula, evaluation of adducts, the assignment and comparison of fragmentation pattern (including dealkylation and dearylation products and bio-transformation products), background annotation, an automated library and database search for identification purposes, including mzCloud (ddMS²), Chemspider, MzVault and Mass List Matches [49]. An overview of the Compound Discoverer workflow and parameters can be found in Figure S4. The output of this is a feature list, which includes accurate mass, retention time, m/z adducts and their areas and intensities. Only features that were five times the intensity of the blank were considered.

Based on Compound Discoverer processing results, the identity of most of the peaks was attributed. In particular, five main classes of compounds, i.e., phytochemical compounds (flavanols, flavanols, isoflavones, calchones, anthocyanidin derivatives, terpenoids and sesquiterpenoids, vitamins, gibberellin plant hormones, metabolites), fatty acids (saturated and unsaturated fatty acids and derivatives), amino acids, organic acids, but also various sugars and sugar derivatives could be identified in chestnut hydro-methanolic extracts. The compounds name, molecular formula, retention time, exact mass and accurate mass of m/z adduct ions, and MS/MS fragment ions in negative ESI mode are shown in Table 3 for bioactive phytochemical compounds, organic acids, amino acids and some metabolites and Table 4 for saturated and unsaturated fatty acids and some derivatives.

Total Ion Current (TIC) and the extracted chromatograms (using a 5 ppm mass accuracy window; negative ion mode, full scan, base peak in the range of 75–1000 m/z) of the main compounds identified in the chestnut methanolic extract were presented in Figure S5 for bioactive phytochemical compounds, Figure S6 for saturated and unsaturated fatty acids and some derivatives, Figure S7 for amino acids and in Figure S8 for gibberellin plant hormones.

The most identified phytochemical compounds were flavonoids, a large group of natural compounds reported in plants, which provide fragrance and taste of the fruits and play an important role for human health due to their high pharmacological activities, mainly attributed to the number of hydroxyl groups attached to base structures of these compounds [50]. Various flavonoids present in the methanolic extract of fruits of different chestnut cultivars have been identified as follows: flavanols (gallocatechin, taxifolin, afzelechin, epiafzelechin) flavanols (luteoforol, dihydrokaempferol, aromadendrin, 3,7-dimethyl quercetin), isoflavones (afromosin (castanin), dihydrogenistein, dalbergioidin, genistein), flavanones (eriodictyol, isoquercetin (quercetin-3-glucoside), quercitrin (quercetin-3-rhamnoside), hyperoside (quercetin-3-galactoside)), chalcones (phloretin and phlorizin) and anthocyanidins derivatives, such as pelargonidin3-*O*-(6-caffeoyl-beta-D-glucoside) [51]. These compounds are associated with many health benefits to humans associated with their bioactive properties, such as anti-inflammatory, anticancer, anti-aging, cardio-protective, antifungal, antibacterial and antiviral properties [52,53].

Table 3. Identification of bioactive phytochemical compounds (flavonoids, chalcones, terpenoids and sesquiterpenoids, gibberellin plant hormones), organic acids, amino acids and some nucleosides in chestnut extract by UHPLC-Q-Exactive high-accuracy analysis of deprotonated precursors and fragment ions of specific components combined with data processing using Compound Discoverer software.

Compound Name	Formula	R.T. (min)	Exact Mass	Accurate Mass [M – H]	Experimental Adduct Ion (m/z)	MS ² Fragments (m/z)			
			Phytochemical Con	npounds					
Flavonoids									
(+)-gallocatechin	$C_{15} H_{14} O_7$	5.72	306.0740	305.0667	305.0668	109.0295, 124.016, 125.0249, 137.0248, 139.0400			
(+)-taxifolin/(–)- taxifolin	C ₁₅ H ₁₂ O ₇	7.44/8.37	304.0583	303.0510	303.0510	273.0412, 125.0241, 259.0611, 178.9977, 151.0028			
afzelechin/(–)- epiafzelechin	$C_{15}H_{14}O_5$	11.40/8.67	274.0841	273.0769	273.0770	229.0867; 205.0864; 187.0758; 166.02628; 137.02335; 97.02821			
(–)- dihydrokaempferol/(+)- (aromadendrin)	C ₁₅ H ₁₂ O ₆	8.09/9.55	288.0634	287.0561	287.0562	259.0618; 243.0665; 201.0554; 151.0034; 125.0242			
3,7-dimethyl quercetin	C ₁₇ H ₁₄ O ₇	12.21	330.0740	329.0667	329.0668	314.03860; 299.01480; 285.0354; 271.02044; 243.0267			
afromosin (castanin)	C ₁₇ H ₁₄ O ₅	11.35	298.0841	297.0769	297.0769	282.05362; 283.06802; 267.03021; 253.04797; 167.04965			
dihydro genistein	C ₁₅ H ₁₂ O ₅	10.57	272.0685	271.0612	271.0613	209.0559; 177.0117; 151.0017; 119.0505; 93.0336			
(+,—)- dalbergioidin/eriodictyol	C ₁₅ H ₁₂ O ₆	8.08/9.54	288.0634	287.0561	287.0562	135.0757; 151.0350; 255.0274; 287.0719; 227.0318			
genistein	$C_{15}H_{10}O_5$	12.61	270.05282	269.0456	269.0457	159.04420; 133.02835; 201.05527; 181.06546; 107.01257			
isoquercetin (quercetin-3- glucoside)	$C_{21}H_{20}O_{12}$	7.72	464.0954	463.0881	463.0883	300.02771; 355.02985; 271.02491; 243.02969; 178.99773; 151.00262			
quercitrin (quercetin- 3-rhamnoside)	$C_{21}H_{20}O_{11}$	8.40	448.1005	447.0932	447.0933	151.0051; 179.0007; 243.0323; 271.0276; 301.03852; 284.0353			
hyperoside (quercetin- 3-galactoside)	$C_{21}H_{20}O_{12}$	8.68	464.0954	463.0881	463.0887	300.02771; 355.02985; 271.02491; 243.02969; 178.99773; 151.00262			
Pelargonidin 3-O-(6-caffeoyl-beta- D-glucoside)	C ₃₀ H ₂₆ O ₁₃	5.60	594.13734	593.1301	593.1304	145.0282; 255.0293; 284.0313; 285.0390			
phloretin	C ₁₅ H ₁₄ O ₅	11.40	274.0841	273.0769	273.0770	179.03509; 167.03498; 125.0244; 123.04515			
phlorizin	C ₂₁ H ₂₄ O ₁₀	8.69	436.1370	435.1297	435.1298	167.0459; 273.0846; 274.0563; 122.0333;			

Compound Name	Formula	R.T. (min)	Exact Mass	Accurate Mass [M – H]	Experimental Adduct Ion (m/z)	MS ² Fragments (m/z)			
Other Phytochemical Compounds									
Gingerol	C ₁₇ H ₂₆ O ₄	15.71	294.1831	293.1759	293.1791	178.0657; 193.0906; 99.0809; 293.1751; 137.0009			
5- hydroxyconiferylalcohol	$C_{10}H_{12}O_4$	7.45	196.0736	195.0663	195.0665	195.0665; 179.0665; 193.0657			
azelaic acid	$C_9H_{16}O_4$	7.64	188.1049	187.0976	187.0967	97.1001; 123.1002; 125.1008			
Dihydro caffeic acid/ hydroxyphenyl lactic acid	$C_9H_{10}O_4$	5.42/5.88	182.0579	181.0507	181.0498	59.0001; 109.3005; 118.9004; 121.1001; 135.3025;			
3,4- dihydroxymandelate	$C_8H_8O_5$	6.32	184.0372	183.0299	183.0290	137.0242; 139.0401; 183.0294;			
cucurbitacin F	$C_{30}H_{46}O_7$	12.00	518.3244	517.3171	517.3170	517.3170; 499.3065; 385.2386			
ursolic and oleanolic acids	C ₃₀ H ₄₈ O ₃	16.10	456.3604	455.3531	455.3531	455.3521; 456.3550			
(S)-abscisic acid	$C_{15}H_{20}O_4$	8.13	264.1362	263.1289	263.1289	152.8462; 219.6015; 203.8293; 263.0201			
geranyl acetate	$C_{12}H_{20}O_2$	11.15	196.1463	195.1391	195.1384	1195.1384; 136.1387; 121.2860; 93.2864			
Sebacic acid	$C_{10}H_{18}O_4$	8.47	202.1205	201.1133	201.1124	110.9001; 139.1134; 183.1038; 201.1127;			
		Gibberellin Com	pounds—Plant Ho	rmones (Phytohormo	nes)				
gibberellin A2-O-beta-D-glucoside	C ₂₅ H ₃₄ O ₁₁	8.43	510.2101	509.2029	509.2029	328.2029, 179.2541			
gibberellin A8	$C_{19}H_{24}O_7$	8.86	364.1522	363.1449	363.1450	275.0321; 118.6022; 160.6709;			
gibberellin A19/gibberellin A36	$C_{20}H_{26}O_{6}$	12.08/15.71	362.1729	361.1657	361.1666	203.1055; 229.1291; 273.0125; 360.8283			
gibberellin A53/gibberellin A14	$C_{20}H_{28}O_5$	10.36/10.74	348.1937	347.1864	347.1862/ 347.1864	189.0372; 329.0731; 347.2285;/ 303.1956; 347.1837			
gibberellin A1 (gibberellic acid)/gibberellin A29/gibberellin A34	C ₁₉ H ₂₄ O ₆	7.80/8.91/9.23	348.1573	347.1500	347.1500/ 347.1499 347.1501	228.5631; 229.2383; 273.1972;/ 259.3782; 303.0891; 347.0805;/			
gibberellin A3	$C_{19}H_{22}O_{6}$	7.35	346.1416	345.1338	345.1344	71.0489; 143.0855; 221.1330			
Gibberellin A12	$C_{20}H_{28}O_4$	10.01	332.1988	331.1915	331.1914	241.1956; 259.2058; 287.2003			
			Organic Aci	ds					
Quinic acid	C7H12O6	3.41	192.0634	191.0561	191.0553	85.0301; 93.0035; 127.0001; 191.0556			
Citric acid	C ₆ H ₈ O ₇	2.14	192.027	191.0197	191.0188	173.0091; 129.0193; 111.0088; 87.00877			
Malic acid	$C_4H_6O_5$	1.47	134.0215	133.0142	133.0130	133.0142; 115.0036; 89.0244; 71.0138			

Table 3. Cont.

Compound Name	Formula	R.T. (min)	Exact Mass	Accurate Mass [M – H]	Experimental Adduct Ion (m/z)	MS ² Fragments (m/z)
			Amino Acid	s		
N-acetyl tryptophan	$C_{13}H_{14}N_2O_3$	7.43	246.1004	245.0932	245.0929	202.0929, 159.0926
N-Acetyl-L- tyrosine	C ₁₁ H ₁₃ NO ₄	6.03	223.0845	222.0772	222.0768	179.0772, 136.1912
N-Acetyl-L- phenylalanine	C ₁₁ H ₁₃ NO ₃	6.86	207.0895	206.0823	206.0815	91.1012; 103.0024; 147.2015;164.2021;
L-Tyrosine methyl ester	C ₁₀ H ₁₃ NO ₃	13.49	195.0895	194.0823	194.0815	133.0657; 194.0817
D-Tryptophan	$C_{11}H_{12}N_2O_2$	6.26	204.0899	203.0826	203.0820	158.0820, 130.0820
L-Tryptophan	$C_{11}H_{12}N_2O_2$	7.42	204.0899	203.0826	203.0820	74.0255; 116.0504; 142.0658; 159.0926; 203.0821
D-Tyrosine	C ₉ H ₁₁ NO ₃	2.55	181.0739	180.0666	180.0658	119.0498; 163.0387; 180.0684
L-Glutamic acid	C ₅ H ₉ NO ₄	1.26	147.0532	146.0459	146.0466	102.0559; 128.0353;
		Other	bioactive compound	ds/metabolites		
Pantothenic acid (Vitamin B5)	$C_9H_{17}NO_5$	4.97	219.1107	218.1034	218.1028	71.0512; 88.0407; 146.0816; 218.1029;
vitamin C	$C_6H_8O_6$	2.64	176.0321	175.0248	175.0238	127.0036; 115.0037; 87.0087; 59.0138
Uridine	$C_9H_{12}N_2O_6$	2.44	244.0695	243.0623	243.0619	109.9196; 200.0670; 140.0354; 152.0355; 243.0621;
Thymidine	$C_{10}H_{14}N_2O_5$	5.16	242.0903	241.0830	241.0828	42.2001; 124.9002; 151.0504; 241.0822;
Guanosine	C ₁₀ H ₁₃ N ₅ O ₅	4.18	283.0917	282.0844	282.0845	108.0201; 133.0154; 150.0421; 282.0844;

Table 3. Cont.

Other phytochemical compounds identified in the chestnut extract are phenol phytochemical, such as gingerol and dihydro caffeic acid with anticancer, anti-inflammatory, anti-fungal, antioxidant, neuroprotective and gastro-protective properties [54], carboxylic acids, such as sebacic and azaleic acids used to treat mild to moderate acne [55] and 3,4-dihydroxymandelate, a compound with a powerful antioxidative potential present in some fruits [56]. Some triterpenes and sesquiterpenes were also identified, among them cucurbitacin F which presents cytotoxic properties [57], ursolic and oleanolic acids with numerous beneficial effects, including anti-oxidative, anti-inflammatory, anticancer and neuroprotective potential [58], abscisic acid, a plant hormone that plays an important role in managing glucose homeostasis in humans [59] and geranyl acetate, a plant metabolite with anti-inflammatory properties [60].

Gibberellin plant hormones represent tetracyclic diterpene acids with significant agricultural importance in plants, regulating seed germination, root and shoot elongation, flowering, and fruit patterning [61]. Of the more than 130 GAs identified so far in plants, fungi and bacteria, gibberellic acid (GA1), GA3, GA4, and GA7 shows bioactive properties, being used as endogenous regulators of plant growth and development [62]. In chestnut extracts were identified bioactive gibberellin GA1 and GA3 which shows antimicrobial properties against phytopathogens and clinical pathogens, thus being a powerful tool for antimicrobial resistance to conventional antibiotics [63], but also other gibberellins, such as GA8, GA12, GA14, GA19, GA29, GA34, GA36, GA53.

Amino acids are essential elements in the synthesis of proteins in the body and help meet the body's needs for growth and health [64]. Regarding amino acids, essential amino acids, such as tryptophan (Trp) and some derivatives like N-acetyl tryptophan (AcTrp)

or N-Acetyl-L-phenylalanine (AcPhe) derived from phenylalanine (Phe) were identified in chestnut extracts. These amino acids are precursors for neurotransmitters, such as serotonin, dopamine, norepinephrine (noradrenaline), epinephrine (adrenaline), but also for the skin pigment melanin [65,66]. In addition, some non-essential amino acids, such as glutamic acid (Glu) and tyrosine (Tyr) and some derivatives like *N*-Acetyl-L-tyrosine (AcTyr) and L-Tyrosine methyl ester (TyMeE) were identified in chestnut extracts, these amino acids representing excitatory neurotransmitters, glutamic acid being the precursor for the synthesis of the inhibitory gamma-aminobutyric acid (GABA) in neurons [67], while tyrosine is also involved in the synthesis of the neurotransmitter dopamine which is converted to catecholamines, such as norepinephrine (noradrenaline) and epinephrine (adrenaline) [68]. Therefore, it can be concluded that chestnut fruits and derived food supplements and functional foods represent very good support for the health of the nervous system and skin.

Essential organic acids for human metabolism, such as citric and malic acids [28] were identified in the extracts of the studied chestnut cultivars, indicating that chestnuts represent a potential source of citric acid which shows antioxidant and anti-inflammatory properties and can prove support for neurodegenerative diseases [69] and malic acid which shows skin-care benefits, improving of physical performance, cardiac function, and control of kidney stones and fibromyalgia [70]. Additionally, quinic acid which represents the starting material for the synthesis of different pharmaceuticals [71] was identified in chestnut extracts.

Vitamins

Other bioactive compounds identified in the chestnut extracts are vitamins, such as pantothenic acid (vitamin B5) which is essential for the release of energy from food, for healthy growth and for the production of antibodies and vitamin C which is an essential nutrient in many multicellular organisms, especially in humans [72], but also some nucleosides, such as uridine, thymidine and guanosine which were correlated with numerous physiological processes in the body and showed antioxidant and antitumor activities [73].

Statistical analysis based on the qualitative data relating to phytochemical (flavonoids, triterpene, phytohormones) and nutritional (amino acids, organic acids, vitamins, nucleosides) UHPLC-MS/MS fingerprints of fruits of different chestnut cultivars harvested in two years was performed in order to discriminate between the chestnut cultivars. As shown in Figure 5a, a clear discrimination can be observed between the fruit of chestnut cultivars harvested in the 2017 harvest year from those harvested in the 2016 year, indicating that climatic conditions (temperature, water availability) play an essential role in the synthesis of fruit phytochemicals and nutrients.

As harvest year plays a crucial role in the synthesis of fruit phytochemicals and nutrients, Hierarchical Cluster Analysis was used as an exploratory tool to assess the dissimilarities between the fruits of different chestnut cultivars harvested in the same year, for example, 2017 (Figure 5b). As observed, fruits of 'Marigoule' and 'Marissard' were grouped separated from the other cultivars, while 'Marsol', 'Bournette' and 'Précoce Migoule' form another group which is clustered separately from 'Maraval'.

Untarget UHPLC-MS/MS analysis coupled with Compound Discoverer processing of the data and Lipid Data Analyzer 2.6, only 10 fatty acids were detected in the studied chestnut cultivars, including five types of saturated fatty acids (lignoceric acid, phytanic acid, stearic acid, palmitic acid and behenic acid) and five types of unsaturated fatty acids (eicosanoic acid, oleic acid, linolenic acid, farnesoic acid and pinellic acid) (Table 4).

Palmitic acid is the most abundant saturated fatty acid in chestnuts, while the unsaturated fatty acids in chestnuts are mainly represented by oleic acid, linoleic acid and α -linolenic acid [64]. **Table 4.** Identification of fatty acids and some derivatives in chestnut extract by UHPLC-Q-Exactive high-accuracy analysis of deprotonated precursors and fragment ions of specific components combined with data processing using Compound Discoverer software.

Compound Name	Formula	R.T. (min)	Exact Mass	Accurate Mass [M – H] ⁻	Experimental Adduct Ion (m/z)	MS ² Fragments (m/z)
		Saturated I	Fatty Acids and I	Derivates		
Lignoceric acid	$C_{24}H_{48}O_2$	18.86	368.3654	367.3582	367.3582	367.3590; 368.3625;
Phytanic acid	$C_{20}H_{40}O_2$	17.12	312.3028	311.2956	311.2956	311.2956, 267.2956
Stearic acid (Octadecanoic acid)	$C_{18}H_{36}O_2$	17.32	284.2715	283.2643	283.2642	283.2636;
Palmitic acid (Hexadecanoic acid)	$C_{16}H_{32}O_2$	16.59	256.2402	255.2330	255.2327	255.2317; 256.2350; 237.3001;
Behenic acid (Docosanoicacid)	$C_{22}H_{44}O_2$	18.40	340.3341	339.3269	339.3267	339.3265; 340.3303;
Dioxo-hydrox(6,9-dioxo-(11R,15S)- dihydroxy-13E-prostenoic acid (6-keto PGE1)	C ₂₀ H ₃₂ O ₆	7.72	368.2199	367.2126	367.2125	143.2541; 205.5478; 124.3256; 269.3254;187.1475;
		Unsaturated	Fatty Acids and	Derivates		
Eicosanoic acid (Arachidic acid)	$C_{20}H_{40}O_2$	17.92	312.3028	311.2956	311.2956	311.2956;
Oleic acid	$C_{18}H_{34}O_2$	16.79	282.2559	281.2486	281.2484	263.3001; 281.2474;
Linolenic acid	$C_{18}H_{30}O_2$	15.88	278.2246	277.2173	277.2172	233.2001; 259.2002; 277.2188
Farnesoic acid	$C_{15}H_{24}O_2$	12.32	236.1776	235.1704	235.1701	235.1701, 191.1704
Pinellic acid	$C_{18}H_{34}O_5$	10.18	330.2406	329.2334	329.2332	329.2332, 285.2332
9Z,11E-octadecadienoic acid (9Z, 11E-Linoleic acid)	$C_{18}H_{32}O_2$	16.30	280.2402	279.2330	279.2328	279.2324; 234.2328
(9S,10S)-9,10-Dihydroxyoctadecanoate	$C_{18}H_{36}O_4$	13.69	316.2614	315.2541	315.2540	315.2540, 271.2541
9,10-dihydroxy-12Z-octadecenoic acid (9,10-DiHOME)/ 12,13-dihydroxy-9Z-octadecenoic acid	$C_{18}H_{34}O_4$	12.89/ 12.41	314.2457	313.2385	313.2385/ 313.2386	171.1081; 201.0922; 277.1702;
2-Hydroperoxy-2,4-octadecadienoic acid/ 8R-hydroperoxy-9Z,12Z-octadecadienoic acid (8-HpODE)/ 13S-hydroperoxy-9Z,11E-octadecadienoic acid (13-HpODE)/ 11S-hydroperoxy-9Z,12Z-octadecadienoic acid (11S-HpODE)	C ₁₈ H ₃₂ O ₄	13.02/ 13.32/ 13.49/ 12.73	312.3028	311.2228	311.2228/ 311.2228/ 311.2229/ 311.2230	223.1041; 205.0112; 211.1208;
11Z-eicosenoic acid (Gondoic acid)/	$C_{20}H_{38}O_2$	17.46	310.2872	309.2799	309.2800	309.2794
9S-hydroperoxy-10E,12Z,15Z- octadecatrienoic acid (9S HpOIrE)/ 13S-hydroperoxy-9Z,11E,15Z- octadecatrienoic acid(13(S)-HpOTrE)	$C_{18}H_{30}O_4$	12.11/ 12.62	310.2144	309.2072	309.2073/ 309.2073	139.0400; 190.1600; 209.1491; 227.1602; 252.0801;
2-Oxooctadecanoic acid (2-oxostearic acid)	$C_{18}H_{34}O_{3}$	14.51	298.2508	297.2435	297.2433	297.2433
12R,13S-epoxy-9Z-octadecenoic acid (12,13-EODE)/ 9S-hydroxy-10E,12Z-octadecadienoic acid (9S-HODE)/ 13S-hydroxy-9Z,11E-octadecadienoic acid(13S-HODE)/ 9R,10S-epoxy-12Z-octadecenoic acid (Coronaric acid/9,10-EODE)	C ₁₈ H ₃₂ O ₃	13.89/ 14.09/ 14.86/ 15.13	296.2351	295.2279	295.2278/ 295.2276/ 295.2277/ 295.2277	195.1461; 277.2102; 295.1675; 171.1026; 277.2171; 295.2276; 195.1112; 277.1801 171.0891; 277.1552;
17-Hydroxylinolenic acid/ 9-oxo-10E,12Z-octadecadienoic acid (9-KODE)/ (9Z)-(13S)-12,13-Epoxyoctadeca-9,11- dienoic acid	C ₁₈ H ₃₀ O ₃	13.47/ 14.66/ 14.87	294.2195	293.2122	293.2123/ 293.2122/ 293.2122	185.0921; 197.1032; 220.1291; 221.1282; 293.8401;
9,12-Dioxododecanoic acid	$C_{12}H_{20}O_4$	6.85	228.1362	227.1289	227.1284	227.1284, 183.1284

Oleic acid is the main component of the cell membrane and cell nucleus and also facilitates the dissolution and absorption of fat-soluble vitamins, such as vitamin E [74]. Together with oleic acid, linolenic acid is involved in the prevention and treatment of cardiovascular and cerebrovascular diseases, thanks to cholesterol elimination [75].

Some saturated fatty acids derivatives like dioxo-hydroxy(6,9-dioxo-(11R,15S)-dihydroxy-13E-prostenoic acid (6-keto PGE1), (R)-10-Hydroxystearate and (R)-2-Hydroxystearate were identified in chestnut methanolic extracts, which can serve as a nanocarrier for ocular delivery of bioactive substances [76]. Furthermore, hydroxy derivatives of octadecenoic and octadecadienoic acids, hydroperoxy derivatives of octadecadienoic and octadecatrienoic acids and epoxy derivatives of octadecenoic acid were identified, these compounds being used to reduce inflammation as a result of metabolic syndrome and cancer [77,78].



Figure 5. (a) PCA based on phytochemical and bioactive UHPLC/MS-MS fingerprints of the fruits of different chestnut cultivars harvested in 2016 and 2017 and (b) Dendrogram of the fruits of different chestnut cultivars harvested in 2017 ('Précoce Migoule'—PM, 'Bournette'—B, 'Marsol'—MS, 'Marissard'—MRS, 'Marigoule'—MG and 'Maraval'—MV).

Statistical analysis based on the qualitative data referring to saturated and unsaturated fatty acids fingerprints of the studied chestnut cultivars harvested in two years was performed in order to distinguish some possible groups. The results indicate that there are big differences in the fatty acid composition of chestnuts in different years, which leads to the identification of two distinct groups corresponding to the harvest years (Figure 6a), which suggest that variation in fatty acid composition depended firstly on the climatic conditions and subsequently on genotype. Lower precipitation and higher temperatures favored the accumulation of fatty acids [79].

HCA analysis indicates that, based on fatty acid and derivatives fingerprints, 'Précoce Migoule' and 'Bournette' form a distinct cluster, while 'Marsol', 'Marissard' and Marigoule are clustered together. 'Maraval' shows a distinct fatty acids profile, being clustered separately (Figure 7b).



Figure 6. (a) PCA based on fatty acids and some derivatives UHPLC/MS-MS fingerprints of the fruits of different chestnut cultivars harvested in 2016 and 2017 and (b) Dendrogram of the fruits of different chestnut cultivars harvested in 2017 ('Précoce Migoule'—PM, 'Bournette'—B, 'Marsol'—MS, 'Marissard'—MRS, 'Marigoule'—MG and 'Maraval'—MV).



Figure 7. (a) Sugar profile of the analyzed chestnut cultivars harvested in 2016 and 2017; (b) Principal Component Analysis (PCA) of the fruits of different chestnut cultivars based on individual phenolic compounds ('Précoce Migoule'—PM, 'Bournette'—B, 'Marsol'—MS, 'Marissard'—MRS, 'Marigoule'—MG and 'Maraval'—MV); (c) PCA analysis based on sugar composition of chestnut fruits harvested in some European countries.

3.4. Sugars Profile of Different Chestnut Cultivars Harvested in Two Years

Carbohydrates are relevant components in chestnuts, with sucrose being an important parameter in assessing the quality of the fruit. Together with sucrose, glucose and fructose are present in significant amounts in chestnuts, the profile of free sugars contributing to the identification of chestnut cultivars. Sugar profile can be influenced by several conditions, such as cultivars, genotypes, environmental factors (climatic conditions, soil characteristics) technical and cultural practices, and harvest time [10].

Large and significant differences (p < 0.0001) in sugar content values were detected among cultivars and harvest years (Table 5). The obtained data were expressed as mean values. The values in the same column followed by different lowercase letters differ significantly depending on the chestnut cultivar and harvest year, according to Duncan's multiple range test at $p \le 0.0001$.

The highest quantity of sugars was observed for 'Bournette' in 2017, followed by 'Marigoule' and 'Précoce Migoule' in the 2016 harvest year (Figure 7a). Sucrose was the most abundant sugar in the analyzed chestnuts with values ranging from 20.34–154.94 g/kg DW, the lower value corresponding to 'Maraval', while the highest value corresponds to 'Bournette' in 2017. The sucrose level of chestnut fruits cultivated in Romania is similar to those grown in Turkey (68.20–174.00 g/kg DW) [10], but lower compared with chestnut fruits cultivated in Italy (2.98–245.09 g/kg DW) [2] and Portugal (40.30–233.00 g/kg DW) [30] and higher compared with chestnut fruits cultivated in Tenerife (Spain) (31.10–99.40 g/kg DW) [29].

Chestnut Cultivar	Code	Fructose	Glucose	Sucrose	Maltose
(D (1), ()	B 2017	5.052 d	9.536 b	154.942 a	2.429 с
Bournette [®] (B)	B 2016	2.645 e	2.99 e	51.967 g	2.268 d
'Précoce Migoule'	PM 2017	14.346 a	14.456 a	51.461 ĥ	2.299 d
(PM)	PM 2016	2.026 f	2.099 g	126.968 c	3.159 a
'Mariaoulo' (MC)	MG 2017	6.85 c	7.42 c	58.85 f	2.400 c
Maligoule (MG)	MG 2016	1.809 g	1.795 h	153.496 b	2.885 b
	MS 2017	1.900 g	2.14 g	63.915 d	2.000 e
Warsol (1915)	MS 2016	1.550 h	1.556 i	62.705 e	2.086 e
'Marissard' (MRS)	MRS 2017	2.055 f	2.308 f	41.047 i	2.792 b
'Maraval' (MV)	MV 2017	11.894 b	5.581 d	20.343 j	1.766 f
R ²		0.9999	0.9999	1.0000	0.9941
F		16979	11345	1073511	186
Pr > F		< 0.0001	< 0.0001	< 0.0001	< 0.0001
Significant		Yes	Yes	Yes	Yes

Table 5. Sugar content in different chestnut cultivars harvested in 2016 and 2017 (g/kg DW).

Data are expressed as means. The lowercase letters in the column represent the different groups resulted from the interactions between the studied factors (cultivar and harvest year), according to Duncan's multiple range test at $p \le 0.05$.

In general, fructose and glucose were quantified in equal amounts in the studied chestnut cultivars, with fructose ranging between 1.55–14.35 g/kg DW and glucose ranging between 1.56–14.46 g/kg DW, the highest contents corresponding to 'Précoce Migoule' harvested in 2017 (Figure 7a). The higher fructose amount compared to the glucose one corresponds to 'Maraval', thus being considered as a functional food for consumers suffering from type 2 diabetes [80]. Our data were higher than those reported by other authors which found glucose and fructose concentrations between not detected and 3.1 g/kg DW for both monosaccharides [81] or between 0.56–2.40 g/kg DW for fructose and 0.49–1.90 g/kg DW for glucose [29], but in the same range as Mert et al. who report fructose between 1.5–8.0 g/kg DW and glucose between 1.77 and 3.16 g/kg DW. It can be concluded that the 2017 harvest year, in which there were higher temperatures and more abundant rainfall, was more favorable to accumulate sugars in 'Bournette', while the 2016 harvest year was more favorable for 'Marigoule' and 'Précoce Migoule' cultivars.

PCA analysis based on sugar profile (sucrose, fructose and glucose) of chestnut fruits grown in the main European chestnut producing countries (Italy, Portugal, Turkey, Spain) and the chestnut cultivated in Romania (Figure 7b), indicate a good correlation between the sugar content of chestnuts and climatic conditions, temperate continental corresponding in Romania and Turkey (around the Black Sea) characterized by an average temperature of 11.94 °C and 566.07 mm precipitation in Turkey and 10.51 °C and 669.15 mm precipitation in Romania, compared with the temperate oceanic climate in Portugal (average temperature of 16.11 °C and 677.93 mm precipitation), the Mediterranean climate in Italy (average temperature of 13.35 °C and 791.32 mm precipitation) and the subtropical climate with some influences from Sahara in Tenerife (Spain) (average temperature of 14.47 °C and 518.24 mm precipitation) (Table S3). The chestnuts from Portugal Italy, Spain and Turkey were grouped in distinct groups, while the chestnuts cultivated in Romania were overlapped on the others.

3.5. Macro and Micronutrients Composition

The content of macro-nutrients (K, Mg, Ca and Na) and micronutrients (Fe, Cu) in fruits of different chestnut cultivars harvested in 2016 and 2017 was determined (Table 6). Furthermore, Ni was investigated as an essential plant micronutrient required for normal plant growth and development. Significant differences (p < 0.0001) in elemental content values were detected among cultivars and harvest years, suggesting that the cultivar and the climatic conditions influence the elemental composition of the chestnut fruits, the soil

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mineral composition being the same for all the studied cultivars. The data were expressed as mean values. The values in the same column followed by different lowercase letters differ significantly depending on the chestnut cultivar and harvest year, according to Duncan's multiple range test at $p \leq 0.0001$.

Table 6. Macro and micronutrients content in different chestnut fruits (*Castanea sativa* Mill) harvested in 2016 and 2017 (mg/100 g DW).

	Ca	Mg	Na	К	Fe	Ni	Cu
PM 2017	29.58 a	77.19 c	30.65 e	2166.46 b	3.09 b	0.90 d	0.60 e
PM 2016	16.87 i	104.81 a	26.39 g	2652.11 a	2.51 c	1.47 a	0.86 b
B 2016	26.08 b	102.88 b	30.79 d	860.02 f	2.15 e	1.03 c	0.64 d
MG 2016	22.92 с	54.68 i	22.65 h	974.86 c	2.42 d	1.38 b	0.98 a
MS 2016	21.90 d	65.56 h	26.57 f	867.16 e	2.10 g	0.71 f	0.75 c
B 2017	17.80 g	77.15 d	172.03 a	710.36 i	2.14 e	0.63 g	0.47 g
MRS 2017	17.15 ĥ	66.42 g	157.21 b	884.42 d	2.12 f	0.51 i	0.60 e
MS 2017	14.01 j	48.62 j	33.17 c	403.56 j	3.43 a	0.21 j	0.54 f
MV 2017	19.81 e	68.45 f	22.48 i	747.02 h	2.09 g	0.81 e	0.46 h
MG 2017	19.70 f	68.69 e	4.63 j	793.65 g	1.89 h	0.57 h	0.31 i
R ²	1.000	1.000	1.000	1.000	1.000	1.000	0.999
F	779,671	8,429,549	121,574,044	14,113,376,066	14,976	14,441	2102
$\Pr > F$	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Significant	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Data are expressed as means the lowercase letters in the column represent the different groups resulting from the interactions between the studied factors (cultivar and harvest year), according to Duncan's multiple range test at $p \le 0.05$.

Concerning the macronutrients, K was the most abundant, ranging between 403.56 mg/100 g DW and 2652.11 mg/100 g DW, followed by Na which ranged between 4.63 mg/100 g DW and 17.20 mg/100 g DW. Calcium contents ranged from 14.01 mg/100 g DW in 'Marsol' to 29.58 mg/100 g DW in 'Précoce Migoule', while Mg content varied between 48.62 mg/100 g in 'Marsol' and 102.88 mg/100 g DW in 'Précoce Migoule'. The obtained macronutrients values are higher than those reported in other studies, for the same cultivar, but cultivated in different geographical regions. For example, the macronutrient content of chestnut cultivar 'Marsol' is lower than that reported for 'Marsol' grown in Croatia (1383.90 mg/100 g for K, 39.20 mg/100 g for Na, 100.70 mg/100 g for Ca and 97.90 mg/100 g for Mg) [6] and the macronutrient content of the chestnut cultivar 'Marigoule' and 'Maraval' is lower or similar to that reported for the same cultivars in Turkey [27]. These differences could be explained by the poor content of the macronutrients in the clay-alluvial soil specific to the foothills outside the Carpathians (Vâlcea county) and the soil from Croatia and Turkey, which are less washed and less acidic due to the corresponding warm climate, and therefore, there is a great availability of these minerals from the soil to plant. With regard to micronutrients, chestnut fruits also contain significant amounts of Fe (1.84-3.43 mg/100 g DW) and Cu (0.31–0.98 mg/100 g DW) which are similar to values found in other studies [6,27], but lower than those obtained by several authors [23,82].

The Ni content in chestnut fruits ranged from 0.21 to 1.47 mg/100g DW, proving that the chestnuts had a sufficient amount of Ni for normal growth and development, in general, the plants' Ni requirement was 0.5 mg per kg of dry weight [83]. Furthermore, the Ni content in chestnut fruits does not exceed the maximum permissible values of 67.9 mg/kg in vegetables for human consumption [84].

The growing concern for a healthy diet rich in nutrients has become an important public health issue and this includes a balanced mineral intake. Potassium is a very significant body mineral, important to cellular and electrical functions, and is one of the main blood minerals called "electrolytes" [85]. Sodium along with potassium helps maintain the proper balance of body fluids, but it also helps to transmit nerve impulses and regulates muscle contraction and relaxation. Magnesium maintains the electrical potential

of nerve tissues and cell membranes and has an important enzymatic role, being involved in the synthesis of proteins, RNA and DNA [11]. Calcium gives rigidity to the skeleton and is an important part of bones and teeth. Ca supplementation is essential for stimulating growth and preventing osteoporosis [86]. Micro-elements also play important roles in health. For example, iron is responsible for the synthesis and catabolism of some nutrients, being the essential components of enzymes involved in these processes. Additionally, iron is involved in transporting oxygen through red blood cell hemoglobin and for that, appropriate supplementation of Fe can effectively prevent anemia [87].

K was quantified in higher amounts in the 'Précoce Migoule' cultivar, while the lower content was observed for the 'Marsol' cultivar. Larger quantities of Na were identified in the 'Bournette' and 'Marissard' chestnut cultivars, while equal amounts of Mg and Ca were quantified in the studied chestnut cultivars (Figure 8a). Micronutrient profiles of the studied chestnut cultivars are similar, with small exceptions, such as Fe being quantified in high amounts in the 'Marsol' cultivar (Figure 8b). Among the studied chestnut cultivars, 'Précoce Migoule' showed the highest mineral content, while the lowest corresponded to 'Marsol'.



Figure 8. Mineral profile of the analyzed chestnut cultivars harvested in 2016 and 2017: (**a**) Ca, Mg, Na and K; (**b**) Fe, Ni and Cu.

Using principal component analysis to classify cultivars according to the main nutrient components, we have found that the two main principal components accumulated more than 60.61% of the variance (Figure 9a). The first principal component (PC1) discriminated chestnut cultivars harvested in 2016 from those harvested in 2017. K, Ca, Mg, Cu and Ni nutrients were distributed towards the chestnut samples harvested in 2016, a year that was less warm compared with 2017 (Table 1), which favors the mobility and absorbance of individual nutrients from the soil, and consequently, the accumulation of minerals from the soil to the plant [88,89]. Na was distributed towards chestnut cultivars harvested in 2017, in which higher amounts of precipitation were recorded, which favors the mobility of Na from soil into the plant.

Because chestnut fruit mineral composition is not only associated with the genotype and climatic conditions but is also related to the mineral composition of the soil where the chestnut trees were grown, we try to discriminate the sweet chestnut fruits cultivated in Romania from those grown in the large chestnut-producing countries from Europa, such as Portugal, Turkey, Spain and Croatia, based on the available literature data [3,6,23,27,83,90] (Figure 9b).

Clear discrimination can be observed for chestnuts harvested in Portugal and Croatia, while the chestnuts harvested in Turkey and Spain are overlapped. The chestnut samples harvested in Romania were partially overlapped on the others, indicating that a rigorous analysis, which takes into account all the variables (genotype, climatic conditions, soil characteristics) is required.



Figure 9. (a) PCA analysis based on mineral composition of fruits of different chestnut cultivars harvested in Romania in 2016 and 2017 ('Précoce Migoule'—PM, 'Bournette'—B, 'Marsol'—MS, 'Marissard'—MRS, 'Marigoule'—MG and 'Maraval'—MV) and (b) PCA analysis based on mineral composition of chestnut fruits harvested in some European countries.

4. Conclusions

This study highlights, for the first time, a comprehensive bioactive characterization of 'Marsol', 'Maraval', 'Bournette', 'Précoce Migoule' and 'Marissard' sweet chestnut cultivars grown at Fruit Growing Research—Extension Station (SCDP) Vâlcea, in Northern Oltenia, Romania in order to provide valuable information for selection of the chestnut cultivar with high quality bioactive characteristics that can be cultivated for the development of different value-added food products with multiple benefits on human health.

Based on total polyphenolic content, total flavonoids, antioxidant activity and individual phenolic compounds profile, no significant differences were observed between the studied cultivars, a higher bioactive potential corresponding to 'Marissard', was followed by 'Précoce Migoule', 'Marigoule' and 'Bournette'. Based on gallic and cinnamic acids and rutin some differences can be observed between the studied cultivars variables, with 'Bournette' showing higher gallic acid content and lower cinnamic acid, while 'Marissard' showed the highest cinnamic acid and rutin amounts. Discriminant analysis indicates a clear discrimination between the chestnut fruits harvested in different years, indicating that climatic conditions play o significant contribution to the synthesis of chestnut fruit's bioactive compounds, warm temperature and drought favoring their accumulation and thus, obtaining a crop rich in bioactive compounds, such as gallic, ellagic, cinnamic, chlorogenic and caffeic acids, (+)-catechin and (-)-epicatechin.

Screening HRMS analysis coupled with data processing analysis using Compound Discoverer software based on an untargeted metabolomics working template allows the identification of other bioactive compounds in chestnut hydro-methanolic extracts, such as phytochemical compounds (flavanols, isoflavones, calchones, anthocyanidin derivatives, terpenoids and sesquiterpenoids, vitamins, gibberellin plant hormones, metabolites), fatty acids (saturated and unsaturated fatty acids and derivatives), amino acids and organic acids which are likely responsible for their antioxidant and anti-inflammatory activities.

Statistical analysis based on the qualitative data referring to phytochemical fingerprints and saturated and unsaturated fatty acids fingerprints indicates a clear discrimination of chestnut fruits harvested in different years, indicating that chestnut's phytochemical and fatty acid fingerprints depended firstly on the climatic conditions and subsequently on genotype. Based on HRMS phytochemical fingerprints, 'Marigoule' and 'Marissard' were grouped separated from the other cultivars, while based on fatty acid and derivatives fingerprints, 'Précoce Migoule' and 'Bournette' form a distinct cluster from the other cultivars. Quantitative data would be very useful for the identification of chestnut cultivars with high phytochemical and fatty acid composition.

Large and significant differences (p < 0.0001) in sugar and mineral composition were detected among cultivars and harvest years. The cultivar and climatic conditions influence the sugar composition, with the highest quantity of sugars being observed for the 'Bournette' cultivar in 2017, which was warmer and rainier compared to 2016, while the 2016 harvest year was more favorable for the accumulation of sugars in 'Marigoule' and 'Précoce Migoule' cultivars. The cultivar and the climatic conditions influence the elemental composition of the chestnut fruits, with the soil mineral composition being the same for all the studied cultivars. The 'Précoce Migoule' cultivar shows higher macro and micronutrient contents, with K being the most abundant nutrient. Larger quantities of Na were identified in 'Bournette' and 'Marissard', while equal amounts of Mg and Ca were quantified in the studied chestnut cultivars.

PCA analysis based on sugar profile (sucrose, fructose and glucose) and mineral composition of chestnut fruits grown in Romania and those cultivated in the main European chestnut producing countries (Italy, Portugal, Turkey, Spain) indicates a good correlation between the sugar and mineral contents of chestnuts and climatic conditions corresponding to the different geographical areas. It can, thus, be concluded that climatic and soil characteristics corresponding to Northern Oltenia, Romania favors the accumulation of sugars and minerals, and thus, it is possible to obtain crops with nutritional characteristics similar to those obtained in the specific area of culture.

Supplementary Materials: The following supporting information can be downloaded at: https://www.action.com/actionals //www.mdpi.com/article/10.3390/separations9030066/s1, Figure S1. Correlation between: (a) Total Polyphenolics with antioxidant activity and (b) Total Flavonoids with antioxidant activity; Figure S2. Variation of the antioxidant activity (AA), Total polyphenolics (TP) and Total Flavonoids (TF) in chestnut fruits of different sweet cultivars; Figure S3. Variation of individual phenolic compounds (gallic acid—GA, cinnamic acid—CA and rutin—R) in chestnut fruits of different sweet cultivars harvested in 2016 and 2017; Figure S4. Untargeted Metabolomics workflow: Find and identify the differences between samples; Figure S5. TIC and the extracted chromatograms of the main phytochemical compounds identified in chestnut methanolic extract; Figure S6. TIC and the extracted chromatograms of the main saturated (a) and unsaturated (b) fatty acids identified in chestnut methanolic extract; Figure S7. TIC and the extracted chromatograms of the main amino acids identified in chestnut methanolic extract; Figure S8. TIC and the extracted chromatograms of the main gibberellin plant hormones identified in chestnut methanolic extract; Table S1. Sums of Growing Degree Hours and Growing Degree Days for the sweet chestnut cultivars; Table S2. Quality performance data for the phenolic acids and flavonoids profiling by UHPLC-MS/MS in negative ionization mode; Table S3. Climatic conditions (temperature and precipitation) in the main European chestnut producing countries and Romania during 2017, 2016 and 2017 harvest year according to https://climateknowledgeportal.worldbank.org/ (accessed on 7 February 2022).

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References

- 1. Míguez-Soto, B.; Fernández-Cruz, J.; Fernández-López, J. Mediterranean and Northern Iberian gene pools of wild *Castanea sativa* Mill. are two differentiated ecotypes originated under natural divergent selection. *PLoS ONE* **2019**, *14*, e0211315. [CrossRef] [PubMed]
- Beccaro, G.L.; Donno, D.; Lione, G.G.; De Biaggi, M.; Gamba, G.; Rapalino, S.; Riondato, I.; Gonthier, P.; Mellano, M.G. Castanea spp. Agrobiodiversity Conservation: Genotype Influence on Chemical and Sensorial Traits of Cultivars Grown on the Same Clonal Rootstock. *Foods* 2020, 9, 1062. [CrossRef] [PubMed]
- 3. Choupina, A.B. Nutritional and health potential of European chestnut. Rev. Ciências Agrárias 2019, 42, 801–807. [CrossRef]
- 4. Akbulut, M.; Bozhuyuk, M.R.; Ercisli, S.; Skender, A.; Sorkheh, K. Chemical Composition of Seed Propagated Chestnut Genotypes from Northeastern Turkey. *Not. Bot. Horti Agrobot.* **2017**, 45, 425–430. [CrossRef]
- 5. Otles, S.; Selek, I. Phenolic compounds and antioxidant activities of chestnut (*Castanea sativa* Mill.) fruits. *Qual. Assur. Saf. Crop. Foods* **2012**, *4*, 199–205. [CrossRef]
- 6. Poljak, I.; Vahčić, N.; Vidaković, A.; Tumpa, K.; Žarković, I.; Idžojtić, M. Traditional Sweet Chestnut and Hybrid Varieties: Chemical Composition, Morphometric and Qualitative Nut Characteristics. *Agronomy* **2021**, *11*, 516. [CrossRef]
- 7. Gonçalves, B.; Borges, O.; Costa, H.S.; Bennett, R.; Santos, M.; Silva, A.P. Metabolite composition of chestnut (*Castanea sativa* Mill.) upon cooking: Proximate analysis, fibre, organic acids and phenolics. *Food Chem.* **2010**, *122*, 154–160. [CrossRef]
- 8. El Khoury, D.; Balfour-Ducharme, S.; Joye, I.J. A Review on the Gluten-Free Diet: Technological and Nutritional Challenges. *Nutrients* **2018**, *10*, 1410. [CrossRef]
- Rodrigues, P.; Ferreira, T.; Nascimento-Gonçalves, E.; Seixas, F.; da Costa, R.M.G.; Martins, T.; Neuparth, M.J.; Pires, M.J.; Lanzarin, G.; Félix, L.; et al. Dietary Supplementation with Chestnut (*Castanea sativa*) Reduces Abdominal Adiposity in FVB/n Mice: A Preliminary Study. *Biomedicines* 2020, *8*, 75. [CrossRef]
- 10. Mert, C.; Ertürk, Ü. Chemical Compositions and Sugar Profiles of Consumed Chestnut Cultivars in the Marmara Region, Turkey. *Not. Bot. Horti Agrobot.* **2017**, *45*, 203–207. [CrossRef]
- 11. Da, L.R.; Silva, B.M. *Natural Bioactive Compounds from Fruits and Vegetables as Health Promoters*; Bentham Science Publishers: Sharjah, United Arab Emirates, 2016.
- 12. Vella, F.M.; Laratta, B.; La Cara, F.; Morana, A. Recovery of bioactive molecules from chestnut (*Castanea sativa* Mill.) by-products through extraction by different solvents. *Nat. Prod. Res.* **2017**, *32*, 1022–1032. [CrossRef] [PubMed]
- 13. Brochard, M.; Correia, P.; Barroca, M.J.; Guiné, R.P.F. Development of a New Pasta Product by the Incorporation of Chestnut Flour and Bee Pollen. *Appl. Sci.* **2021**, *11*, 6617. [CrossRef]
- 14. Pinto, D.; Rodrigues, F.; Braga, N.; Santos, J.; Pimentel, F.B.; Palmeira-De-Oliveira, A.; Oliveira, M.B.P.P. The *Castanea sativa* bur as a new potential ingredient for nutraceutical and cosmetic outcomes: Preliminary studies. *Food Funct.* **2017**, *8*, 201–208. [CrossRef] [PubMed]
- 15. Silva, V.; Falco, V.; Dias, M.I.; Barros, L.; Silva, A.; Capita, R.; Alonso-Calleja, C.; Amaral, J.S.; Igrejas, G.; Ferreira, I.C.F.R.; et al. Evaluation of the Phenolic Profile of *Castanea sativa* Mill. By-Products and Their Antioxidant and Antimicrobial Activity against Multiresistant Bacteria. *Antioxidants* 2020, *9*, 87. [CrossRef] [PubMed]
- 16. You, T.T.; Zhou, S.K.; Wen, J.L.; Ma, C.; Xu, F. Chemical Composition, Properties, and Antimicrobial Activity of the Water-Soluble Pigments from Castanea mollissima Shells. *J. Agric. Food Chem.* **2014**, *62*, 1936–1944. [CrossRef] [PubMed]
- Morales, A.; Gullón, B.; Dávila, I.; Eibes, G.; Labidi, J.; Gullón, P. Optimization of alkaline pretreatment for the co-production of biopolymer lignin and bioethanol from chestnut shells following a biorefinery approach. *Ind. Crops Prod.* 2018, 124, 582–592. [CrossRef]
- Pinto, D.; Cádiz-Gurrea, M.d.I.L.; Vallverdú-Queralt, A.; Delerue-Matos, C.; Rodrigues, F. *Castanea sativa* shells: A review on phytochemical composition, bioactivity and waste management approaches for industrial valorization. *Food Res. Int.* 2021, 144, 110364. [CrossRef]
- 19. Corona, P.; Frangipane, M.T.; Moscetti, R.; Feudo, G.L.; Castellotti, T.; Massantini, R. Chestnut Cultivar Identification through the Data Fusion of Sensory Quality and FT-NIR Spectral Data. *Foods* **2021**, *10*, 2575. [CrossRef]
- Barreira, J.C.M.; Casal, S.; Ferreira, I.C.F.R.; Oliveira, M.B.P.P.; Pereira, J.A. Nutritional, Fatty Acid and Triacylglycerol Profiles of *Castanea sativa* Mill. Cultivars: A Compositional and Chemometric Approach. J. Agric. Food Chem. 2009, 57, 2836–2842. [CrossRef]
- Barreira, J.C.M.; Casal, S.; Ferreira, I.C.F.R.; Peres, A.M.; Pereira, J.A.; Oliveira, M.B.P.P. Chemical characterization of chestnut cultivars from three consecutive years: Chemometrics and contribution for authentication. *Food Chem. Toxicol.* 2012, 50, 2311–2317. [CrossRef]
- 22. Peña-Méndez, E.M.; Hernández-Suárez, M.; Díaz-Romero, C.; Rodríguez-Rodríguez, E. Characterization of various chestnut cultivars by means of chemometrics approach. *Food Chem.* **2008**, *107*, 537–544. [CrossRef]
- De Vasconcelos, M.C.B.M.; Bennett, R.N.; Rosa, E.A.S.; Ferreira-Cardoso, J.V. Composition of European chestnut (*Castanea sativa* Mill.) and association with health effects: Fresh and processed products. *J. Sci. Food Agric.* 2010, *90*, 1578–1589. [CrossRef] [PubMed]
- 24. Mota, M.; Pinto, T.; Vilela, A.; Marques, T.; Borges, A.; Caço, J.; Ferreira-Cardoso, J.; Raimundo, F.; Gomes-Laranjo, J. Irrigation positively affects the chestnut's quality: The chemical composition, fruit size and sensory attributes. *Sci. Hortic.* **2018**, *238*, 177–186. [CrossRef]
- 25. Furones-Pérez, P.; Fernández-López, J. Morphological and phenological description of 38 sweet chestnut cultivars (*Castanea sativa* Miller) in a contemporary collection. *Spanish J. Agric. Res.* **2009**, *7*, 829–843. [CrossRef]

- De Vasconcelos, M.D.C.B.M.; Bennett, R.N.; Rosa, E.A.S.; Cardoso, J.V.F. Primary and secondary metabolite composition of kernels from three cultivars of Portuguese chestnut (*Castanea sativa* Mill.) at different stages of industrial transformation. *J. Agric. Food Chem.* 2007, 55, 3508–3516. [CrossRef]
- Ertürk, Ü.; Mert, C.; Soylu, A. Chemical composition of fruits of some important chestnut cultivars. *Braz. Arch. Biol. Technol.* 2006, 49, 183–188. [CrossRef]
- Delgado, T.; Ramalhosa, E.; Pereira, J.A.; Casal, S. Organic acid profile of chestnut (*Castanea sativa* Mill.) as affected by hot air convective drying. *Int. J. Food Prop.* 2018, 21, 557–565. [CrossRef]
- Suárez, M.H.; Galdón, B.R.; Mesa, D.R.; Romero, C.D.; Rodríguez, E.R. Sugars, Organic Acids and Total Phenols in Varieties of Chestnut Fruits from Tenerife (Spain). *Food Nutr. Sci.* 2012, *3*, 705–715. [CrossRef]
- 30. Barreira, J.C.M.; Pereira, J.A.; Oliveira, M.B.P.P.; Ferreira, I.C.F.R. Sugars Profiles of Different Chestnut (*Castanea sativa* Mill.) and Almond (*Prunus dulcis*) Cultivars by HPLC-RI. *Plant Foods Hum. Nutr.* **2010**, *65*, 38–43. [CrossRef]
- 31. Leichtweis, M.G.; Oliveira, M.B.P.P.; Ferreira, I.C.F.R.; Pereira, C.; Barros, L. Sustainable Recovery of Preservative and Bioactive Compounds from Food Industry Bioresidues. *Antioxidants* **2021**, *10*, 1827. [CrossRef]
- Chira, D.; Bolea, V.; Botu, M.; Giorgi, N.; Juveloiu, E. Sweet chestnut (*Castanea sativa* Mill.) forest in Romania: Distribution, current state, management and research activities. *Revista Silvicultură Cinegetică* 2013, 33, 15–20.
- Cosmulescu, S.; Trandafir, I.; Nour, V.; Botu, M. Physical and compositional characteristics of chestnut fruits. Rom. J. Hortic. 2020, 1, 51–58. [CrossRef]
- Ciucure, C.T.; Geană, E.I. Phenolic compounds profile and biochemical properties of honeys in relationship to the honey floral sources. *Phytochem. Anal.* 2019, 30, 481–492. [CrossRef] [PubMed]
- 35. Geană, E.-I.; Ciucure, C.T.; Ionete, R.E.; Ciocârlan, A.; Aricu, A.; Ficai, A.; Andronescu, E. Profiling of Phenolic Compounds and Triterpene Acids of Twelve Apple (*Malus domestica* Borkh.) Cultivars. *Foods* **2021**, *10*, 267. [CrossRef] [PubMed]
- Geană, E.-I.; Ciucure, C.T.; Costinel, D.; Ionete, R.E. Evaluation of honey in terms of quality and authenticity based on the general physicochemical pattern, major sugar composition and δ¹³C signature. *Food Control* 2020, 109, 106919. [CrossRef]
- Chiriac, E.R.; Chiţescu, C.L.; Sandru, C.; Geană, E.-I.; Lupoae, M.; Dobre, M.; Borda, D.; Gird, C.E.; Boscencu, R. Comparative study of the bioactive properties and elemental composition of red clover (*Trifolium pratense*) and alfalfa (*Medicago sativa*) sprouts during germination. *Appl. Sci.* 2020, 10, 7249. [CrossRef]
- 38. Daramola, B. Preliminary investigation on antioxidant interactions between bioactive components of Solanum anguivi and Capsicum annuum. *J. Food Sci. Technol.* **2018**, *55*, 3827–3832. [CrossRef]
- Šamec, D.; Karalija, E.; Šola, I.; Vujčić Bok, V.; Salopek-Sondi, B. The Role of Polyphenols in Abiotic Stress Response: The Influence of Molecular Structure. *Plants* 2021, 10, 118. [CrossRef]
- Zavalloni, C.; Andresen, J.A.; Flore, J.A. Phenological Models of Flower Bud Stages and Fruit Growth of 'Montmorency' Sour Cherry Based on Growing Degree-day Accumulation. J. Am. Soc. Hortic. Sci. 2006, 131, 601–607. [CrossRef]
- 41. Dinis, L.T.; Peixoto, F.; Pinto, T.; Costa, R.; Bennett, R.N.; Gomes-Laranjo, J. Study of morphological and phenological diversity in chestnut trees ('Judia' variety) as a function of temperature sum. *Environ. Exp. Bot.* **2011**, *70*, 110–120. [CrossRef]
- 42. Santos, J.A.; Costa, R.; Fraga, H. Climate change impacts on thermal growing conditions of main fruit species in Portugal. *Clim. Change* **2017**, *140*, 273–286. [CrossRef]
- Soifoini, T.; Donno, D.; Jeannoda, V.; Rakoto, D.D.; Msahazi, A.; Farhat, S.M.M.; Oulam, M.Z.; Beccaro, G.L. Phytochemical composition, antibacterial activity, and antioxidant properties of the artocarpus altilis fruits to promote their consumption in the comoros islands as potential health-promoting food or a source of bioactive molecules for the food industry. *Foods* 2021, 10, 2136. [CrossRef] [PubMed]
- Ruwizhi, N.; Aderibigbe, B.A. Cinnamic Acid Derivatives and Their Biological Efficacy. Int. J. Mol. Sci. 2020, 21, 5712. [CrossRef] [PubMed]
- 45. Camilla Bergonzi, M.; Heard, C.M.; Lupaescu, A.-V.; Iavorschi, M.; Covasa, M. The Use of Bioactive Compounds in Hyperglycemia and Amyloid Fibrils-Induced Toxicity in Type 2 Diabetes and Alzheimer's Disease. *Pharmaceutics* **2022**, *14*, 235. [CrossRef]
- 46. Ganeshpurkar, A.; Saluja, A.K. The Pharmacological Potential of Rutin. *Saudi Pharm. J.* 2017, 25, 149–164. [CrossRef]
- Kahkeshani, N.; Farzaei, F.; Fotouhi, M.; Alavi, S.S.; Bahramsoltani, R.; Naseri, R.; Momtaz, S.; Abbasabadi, Z.; Rahimi, R.; Farzaei, M.H.; et al. Pharmacological effects of gallic acid in health and disease: A mechanistic review. *Iran. J. Basic Med. Sci.* 2019, 22, 225–237. [CrossRef]
- Esposito, T.; Celano, R.; Pane, C.; Piccinelli, A.L.; Sansone, F.; Picerno, P.; Zaccardelli, M.; Aquino, R.P.; Mencherini, T. Chestnut (*Castanea sativa* Miller.) Burs Extracts and Functional Compounds: UHPLC-UV-HRMS Profiling, Antioxidant Activity, and Inhibitory Effects on Phytopathogenic Fungi. *Molecules* 2019, 24, 302. [CrossRef]
- Hohrenk, L.L.; Itzel, F.; Baetz, N.; Tuerk, J.; Vosough, M.; Schmidt, T.C. Comparison of Software Tools for Liquid Chromatography-High-Resolution Mass Spectrometry Data Processing in Nontarget Screening of Environmental Samples. *Anal. Chem.* 2020, 92, 1898–1907. [CrossRef]
- Ruiz-Cruz, S.; Chaparro-Hernández, S.; Hernández-Ruiz, K.L.; Cira-Chávez, L.A.; Estrada-Alvarado, M.I.; Gassos Ortega, L.E.; De, J.; Ornelas-Paz, J.; Lopez Mata, M.A. Flavonoids: Important Biocompounds in Food. In *Flavonoids From Biosynthesis to Human Health*; Intechopen: London, UK, 2017. [CrossRef]
- Dias, M.C.; Pinto, D.C.G.A.; Silva, A.M.S. Plant Flavonoids: Chemical Characteristics and Biological Activity. *Molecules* 2021, 26, 5377. [CrossRef]

- Jucá, M.M.; Filho, F.M.S.C.; de Almeida, J.C.; Mesquita, D.d.S.; Barriga, J.R.d.M.; Dias, K.C.F.; Barbosa, T.M.; Vasconcelos, L.C.; Leal, L.K.A.M.; Ribeiro, J.E.; et al. Flavonoids: Biological activities and therapeutic potential. *Nat. Prod. Res.* 2018, 34, 692–705. [CrossRef]
- 53. Fraga, C.G.; Croft, K.D.; Kennedy, D.O.; Tomás-Barberán, F.A. The effects of polyphenols and other bioactives on human health. *Food Funct.* **2019**, *10*, 514–528. [CrossRef] [PubMed]
- Ghasemzadeh, A.; Jaafar, H.Z.E.; Rahmat, A.; Feliciano, A.S.; Santos-Buelga, C. Variation of the Phytochemical Constituents and Antioxidant Activities of *Zingiber officinale* var. rubrum Theilade Associated with Different Drying Methods and Polyphenol Oxidase Activity. *Molecules* 2016, 21, 780. [CrossRef] [PubMed]
- 55. Vargas-Diez, E.; Hofmann, M.A.; Bravo, B.; Malgazhdarova, G.; Katkhanova, O.A.; Yutskovskaya, Y. Azelaic Acid in the Treatment of Acne in Adult Females: Case Reports. *Skin Pharmacol. Physiol.* **2014**, *27*, 18–25. [CrossRef]
- 56. Zadernowski, R.; Czaplicki, S.; Naczk, M. Phenolic acid profiles of mangosteen fruits (*Garcinia mangostana*). Food Chem. 2009, 112, 685–689. [CrossRef]
- Méndez-Cuesta, C.A.; Laura, A.; Campos, E.; Sánchez, D.S.; González, C.P.; Gutiérrez, S.P. Cytotoxic and Antitumoral Activities of Compounds Isolated from Cucurbitaceae Plants. In *Pharmacognosy Medicinal Plants*; IntechOpen: London, UK, 2018. [CrossRef]
- Gudoityte, E.; Arandarcikaite, O.; Mazeikiene, I.; Bendokas, V.; Liobikas, J. Ursolic and Oleanolic Acids: Plant Metabolites with Neuroprotective Potential. *Int. J. Mol. Sci.* 2021, 22, 4599. [CrossRef] [PubMed]
- 59. Zocchi, E.; Hontecillas, R.; Leber, A.; Einerhand, A.; Carbo, A.; Bruzzone, S.; Tubau-Juni, N.; Philipson, N.; Zoccoli-Rodriguez, V.; Sturla, L.; et al. Abscisic Acid: A Novel Nutraceutical for Glycemic Control. *Front. Nutr.* **2017**, *4*, 24. [CrossRef]
- 60. Gonçalves, M.J.; Cruz, M.T.; Tavares, A.C.; Cavaleiro, C.; Lopes, M.C.; Canhoto, J.; Salgueiro, L. Composition and biological activity of the essential oil from Thapsia minor, a new source of geranyl acetate. *Ind. Crop. Prod.* **2012**, *35*, 166–171. [CrossRef]
- 61. Binenbaum, J.; Weinstain, R.; Shani, E. Gibberellin Localization and Transport in Plants. *Trends Plant Sci.* 2018, 23, 410–421. [CrossRef]
- 62. Zhang, C.; Tanabe, K.; Tani, H.; Nakajima, H.; Mori, M.; Sakuno, E. Biologically Active Gibberellins and Abscisic Acid in Fruit of Two Late-maturing Japanese Pear Cultivars with Contrasting Fruit Size. J. Am. Soc. Hortic. Sci. 2007, 132, 452–458. [CrossRef]
- 63. Toner, P.; Nelson, D.; Rao, J.R.; Ennis, M.; Moore, J.E.; Schock, B. Antimicrobial properties of phytohormone (gibberellins) against phytopathogens and clinical pathogens. *Access Microbiol.* **2021**, *3*, 278. [CrossRef]
- 64. Zhou, P.; Zhang, P.; Guo, M.; Li, M.; Wang, L.; Adeel, M.; Shakoor, N.; Rui, Y. Effects of age on mineral elements, amino acids and fatty acids in Chinese chestnut fruits. *Eur. Food Res. Technol.* **2021**, 247, 2079–2086. [CrossRef]
- Akram, M.; Daniyal, M.; Ali, A.; Zainab, R.; Muhammad, S.; Shah, A.; Munir, N.; Tahir, I.M. Role of Phenylalanine and Its Metabolites in Health and Neurological Disorders. In *Synucleins—Biochemisty and Role in Diseases*; IntechOpen: London, UK, 2020. [CrossRef]
- 66. Höglund, E.; Øverli, Ø.; Winberg, S. Tryptophan metabolic pathways and brain serotonergic activity: A comparative review. *Front. Endocrinol.* **2019**, *10*, 158. [CrossRef] [PubMed]
- 67. James, S.P.; Bondugji, D. Gamma-Aminobutyric Acid (GABA) and the Endocannabinoids: Understanding the Risks and Opportunities. In *Natural Drugs from Plants*; IntechOpen: London, UK, 2021. [CrossRef]
- 68. Fernstrom, J.D.; Fernstrom, M.H. Tyrosine, Phenylalanine, and Catecholamine Synthesis and Function in the Brain. J. Nutr. 2007, 137, 1539S–1547S. [CrossRef]
- 69. Abdel-Salam, O.M.E.; Youness, E.R.; Mohammed, N.A.; Morsy, S.M.Y.; Omara, E.A.; Sleem, A.A. Citric Acid Effects on Brain and Liver Oxidative Stress in Lipopolysaccharide-Treated Mice. J. Med. Food **2014**, 17, 588. [CrossRef]
- Qiang, F. Effect of Malate-oligosaccharide Solution on Antioxidant Capacity of Endurance Athletes. Open Biomed. Eng. J. 2015, 9, 326. [CrossRef] [PubMed]
- 71. Fernández, S.; Ferrero, M. Strategies for the Synthesis of 19-nor-Vitamin D Analogs. Pharmacology 2020, 13, 159. [CrossRef]
- 72. Devaki, S.J.; Raveendran, R.L. Vitamin C: Sources, Functions, Sensing and Analysis. In *Vitamin C*; InTech: Rijeka, Croatia, 2017. [CrossRef]
- Guo, S.; Duan, J.A.; Zhang, Y.; Qian, D.; Tang, Y.; Zhu, Z.; Wang, H.; McPhee, D.J. Contents Changes of Triterpenic Acids, Nucleosides, Nucleobases, and Saccharides in Jujube (*Ziziphus jujuba*) Fruit During the Drying and Steaming Process. *Molecules* 2015, 20, 22329–22340. [CrossRef] [PubMed]
- 74. Reboul, E. Vitamin E intestinal absorption: Regulation of membrane transport across the enterocyte. *IUBMB Life* **2019**, *71*, 416–423. [CrossRef]
- 75. Visioli, F.; Poli, A. Fatty Acids and Cardiovascular Risk. Evidence, Lack of Evidence, and Diligence. *Nutrients* **2020**, *12*, 3782. [CrossRef]
- 76. Hou, Y.; Zhang, F.; Lan, J.; Sun, F.; Li, J.; Li, M.; Song, K.; Wu, X. Ultra-small micelles based on polyoxyl 15 hydroxystearate for ocular delivery of myricetin: Optimization, in vitro, and in vivo evaluation. *Drug Deliv.* **2019**, *26*, 158–167. [CrossRef]
- 77. Vangaveti, V.N.; Jansen, H.; Kennedy, R.L.; Malabu, U.H. Hydroxyoctadecadienoic acids: Oxidised derivatives of linoleic acid and their role in inflammation associated with metabolic syndrome and cancer. *Eur. J. Pharmacol.* 2016, 785, 70–76. [CrossRef] [PubMed]
- 78. Selvaraj, J. Fatty Acids and Their Analogues as Anticancer Agents. Fatty Acids 2017, 21, 72–86. [CrossRef]
- 79. Chehade, L.A.; Angelini, L.G.; Tavarini, S. Genotype and Seasonal Variation Affect Yield and Oil Quality of Safflower (*Carthamus tinctorius* L.) under Mediterranean Conditions. *Agronomy* **2022**, *12*, 122. [CrossRef]

- 80. Lamothe, L.M.; Lê, K.A.; Samra, R.A.; Roger, O.; Green, H.; Macé, K. The scientific basis for healthful carbohydrate profile. *Crit. Rev. Food Sci. Nutr.* **2017**, *59*, 1058–1070. [CrossRef] [PubMed]
- Míguelez, J.d.l.M.; Bernárdez, M.M.; Queijeiro, J.M.G. Composition of varieties of chestnuts from Galicia (Spain). *Food Chem.* 2004, 84, 401–404. [CrossRef]
- 82. Borges, O.; Gonçalves, B.; de Carvalho, J.L.S.; Correia, P.; Silva, A.P. Nutritional quality of chestnut (*Castanea sativa* Mill.) cultivars from Portugal. *Food Chem.* **2008**, *106*, 976–984. [CrossRef]
- 83. Matraszek, R.; Hawrylak-Nowak, B.; Chwil, S.; Chwil, M. Macronutrient composition of nickel-treated wheat under different sulfur concentrations in the nutrient solution. *Environ. Sci. Pollut. Res.* 2016, 23, 5902–5914. [CrossRef]
- Mensah, E.; Kyei-Baffour, N.; Ofori, E.; Obeng, G. Influence of Human Activities and Land Use on Heavy Metal Concentrations in Irrigated Vegetables in Ghana and Their Health Implications. In *Appropriate Technologies for Environmental Protection in the Developing World*; Sel. Pap. from ERTEP 2007; Springer: Dordrecht, The Netherlands, 2009; pp. 9–14. [CrossRef]
- 85. Udensi, U.K.; Tchounwou, P.B. Potassium Homeostasis, Oxidative Stress, and Human Disease. *Int. J. Clin. Exp. Physiol.* **2017**, *4*, 111. [CrossRef]
- 86. Sunyecz, J.A. The use of calcium and vitamin D in the management of osteoporosis. Ther. Clin. Risk Manag. 2008, 4, 827. [CrossRef]
- 87. Abbaspour, N.; Hurrell, R.; Kelishadi, R. Review on iron and its importance for human health. J. Res. Med. Sci. 2014, 19, 164.
- 88. Da Silva, E.C.; De Albuquerque, M.B.; Dias, A.; Neto, A.; Dias, C.; Junior, S. Drought and Its Consequences to Plants—From Individual to Ecosystem. In *Responses of Organisms to Water Stress*; IntechOpen: London, UK, 2013. [CrossRef]
- Freitas, T.R.; Santos, J.A.; Silva, A.P.; Fraga, H. Influence of Climate Change on Chestnut Trees: A Review. *Plants* 2021, 10, 1463. [CrossRef] [PubMed]
- De Vasconcelos, M.d.C.B.M.; Nunes, F.; Viguera, C.G.; Bennett, R.N.; Rosa, E.A.S.; Ferreira-Cardoso, J.V. Industrial processing effects on chestnut fruits (*Castanea sativa* Mill.) 3. Minerals, free sugars, carotenoids and antioxidant vitamins. *Int. J. Food Sci. Technol.* 2010, 45, 496–505. [CrossRef]