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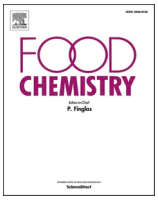

Predicting the effects of *in-vitro* digestion in the bioactivity and bioaccessibility of antioxidant compounds extracted from chestnut shells by supercritical fluid extraction – A metabolomic approach

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ABSTRACT

Chestnut (*Castanea sativa*) shells (CS) are an undervalued antioxidant-rich by-product. This study explores the impact of *in-vitro* digestion on the bioaccessibility, bioactivity, and metabolic profile of CS extract prepared by Supercritical Fluid Extraction, aiming its valorization for nutraceutical applications. The results demonstrated significantly ($p < 0.05$) lower phenolic concentrations retained after digestion (38.57 μg gallic acid equivalents/mg dry weight (DW)), reaching 30% of bioaccessibility. The CS extract showed antioxidant/antiradical, hypoglycemic, and neuroprotective properties after *in-vitro* digestion, along with upmodulating effects on antioxidant enzymes activities and protection against lipid peroxidation. The metabolic profile screened by LC-ESI-LTQ-Orbitrap-MS proved the biotransformation of complex phenolic acids, flavonoids, and tannins present in the undigested extract (45.78 μg/mg DW of total phenolic concentration) into hydroxybenzoic, phenylpropanoic, and phenylacetic acids upon digestion (35.54 μg/mg DW). These findings sustain the valorization of CS extract as a promising nutraceutical ingredient, delivering polyphenols with proven bioactivity even after *in-vitro* digestion.

1. Introduction

Chestnut (*Castanea sativa* Mill.), a widely disseminated crop in southern Europe, is an added value resource in producing countries due to its nutrients-rich fruits (Pinto, Cádiz-Gurrea, Garcia, et al., 2021). Beyond nuts, a large amount of by-products are generated during chestnut farming and processing. Shells are the principal agro-industrial by-product generated by the chestnut production chain (Pinto, Cádiz-Gurrea, Garcia et al., 2021; de Vasconcelos et al., 2010), being described as a rich source of valuable molecules, namely phenolic compounds, vitamin E, and amino acids, with outstanding health benefits, including antioxidant, anti-inflammatory, antihyperlipidemic, and antimicrobial effects (Pinto, Silva, et al., 2021; Pinto, Vieira, et al., 2021; Pinto, Cádiz-Gurrea, Vallverdú-Queralt, et al., 2021; Lameirão et al., 2020; Pinto, Cádiz-Gurrea, & Garcia, et al., 2021). The disposal of this agro-residue entails negative environmental impacts, reinforcing the eminent importance of valorizing it as a novel active ingredient for industrial purposes and, simultaneously, preventing its harmful effects on the environment (Pinto, Cádiz-Gurrea, Garcia, et al., 2021; Pinto et al., 2020). The latest research has proven the *in-vitro* and *in-vivo* antioxidant

Abbreviations: AAE, ascorbic acid equivalents; ABTS, 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid); AChE, acetylcholinesterase; CAT, catalase; CE, catechin equivalents; CoA, coenzyme A; CS, chestnut shells; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DW, dry weight; FRAP, ferric reducing antioxidant power; FSE, ferrous sulfate equivalents; GAE, gallic acid equivalents; GSH-Px, glutathione peroxidase; IC_{50}, half-maximal inhibitory concentration; LC-ESI-LTQ-Orbitrap-MS, liquid chromatography coupled to Orbitrap-mass spectrometry; LPO, lipid peroxidation; MAE, microwave-assisted extraction; MDA, malondialdehyde; ORAC, oxygen radical absorbance capacity; PC, principal component; PCA, principal component analysis; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSM, response surface methodology; SFE, supercritical fluid extraction; SOD, superoxide dismutase; TE, Trolox equivalents; TFC, total flavonoid content; TPC, total phenolic content.

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efficacy of chestnut shells (CS), as well as their ability to improve antioxidant enzymes' activities and reduce lipid peroxidation (LPO) in rats (Pinto, Almeida, et al., 2023; Pinto, Silva, et al., 2021; Pinto, Vieira, et al., 2021; Lameirão et al., 2020; Pinto et al., 2020). The CS bioactivity was closely ascribed to its phenolic composition (Pinto, Almeida, et al., 2023; Pinto, Silva, et al., 2021; Pinto, Vieira, et al., 2021; Lameirão et al., 2020; Pinto et al., 2020). Hence, the extraction of phenolic compounds from CS may be a new sustainable alternative to this undervalued agro-waste, encouraging its use as a potential nutraceutical ingredient for the development of functional foods.

Currently, the search for efficient and clean extraction techniques is a hot topic in the food research field. For instance, supercritical fluid extraction (SFE) has gained ground as an alternative technology committed to the sustainable recovery of bioactive compounds from agro-industrial wastes (Pinto, Cádiz-Gurrea, Vallverdú-Queralt, et al., 2021; Pinto et al., 2020). Several factors influence the performance of SFE, including temperature, time, pressure, and solid–liquid ratio (Pinto, Cádiz-Gurrea, Vallverdú-Queralt, et al., 2021; Pinto et al., 2020). Pinto et al. (2020) optimized the extraction of phenolic compounds from CS by SFE using a response surface methodology (RSM). In addition to CO_2 used as a solvent to enable the extraction of low polar molecules without additional clean-up procedures, ethanol was employed as a co-solvent to improve the solubility of polar molecules and enhance the phenolics recovery yield. The optimal extract was obtained at 60 °C, 350 bar, and 15% of ethanol (co-solvent) and revealed extraordinary antioxidant/antiradical and antimicrobial properties ascribed to the phenolic acids (ellagic acid and caffeic acid derivative), flavonoids (catechins) and procyanidin polymers identified (Pinto, Cádiz-Gurrea, Garcia, et al., 2021; Pinto et al., 2020). The safety of the extract was proven up to 100 μg/mL in two intestinal cell lines (Pinto et al., 2020). This study proposed the valorization of CS extract prepared by SFE as a new active ingredient for nutraceuticals, proving its bioactivity and safety. Nevertheless, the *in-vitro* digestibility of the antioxidants-rich extract from CS and its impact on the phenolic composition and bioactivity have not been evaluated. In fact, during human digestion, pH, temperature, digestive enzymes, and intestinal microbiota directly affect the bioaccessibility of micronutrients (such as vitamins and phenolic compounds) and, consequently, their bioactivity, emphasizing the complexity of the *in-vivo* environment (Hu et al., 2023). Therefore, the implementation of *in-vitro* models that mimic the physiological conditions of the human digestive tract is increasing among the scientific community as robust and useful tools for predicting the impact of digestion on phenolic compounds (Hu et al., 2023; Pinto, Silva, et al., 2023). Although previous research has proven the efficacy of SFE in the recovery of phenolics-rich extracts from CS (Pinto et al., 2020), no studies have demonstrated its bioactivity after gastrointestinal digestion. The present study aims to evaluate the effects of *in-vitro* gastrointestinal digestion on total phenolic and flavonoid contents (TPC and TFC, respectively), antioxidant/antiradical properties, scavenging activity against reactive oxygen and nitrogen species (ROS and RNS, respectively), antioxidant enzymes activities, LPO, and inhibition of acetylcholinesterase (AChE) and α-amylase activities of the CS extract prepared by SFE, considering valorizing it as an active ingredient for nutraceutical products. The metabolic profile was assessed by liquid chromatography coupled to Orbitrap-mass spectrometry (LC-ESI-LTQ-Orbitrap-MS) targeted on phenolic compounds. The recovery and bioaccessibility of phenolic compounds were estimated after each digestion phase. Notably, this paper provides, for the first time, new insights into the phenolic profile and bioactivity of SFE extract from CS digested in an *in-vitro* simulated model, proposing their potential industrial valorization as raw material for nutraceutical industry.

2. Materials and methods

2.1. Chemicals

All chemicals, solvents, and standards were of analytical reagent grade, used as received or dried by standard procedures and acquired from commercial sources. The standards used for metabolomic analyses were supplied as follows: 2,5-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3-hydroxyphenylacetic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(2,4-dihydroxyphenyl)propionic acid, 3,4-dihydroxyhydrocinnamic acid (also known as dihydrocaffeic acid), 3,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, benzoic acid, caffeic acid, catechol, chlorogenic acid, cinnamic acid, *o*-coumaric acid, *m*-coumaric acid, ellagic acid, enterodiol, enterolactone, gallic acid, hippuric acid, homovanillic acid, phenylacetic acid, protocatechuic acid, pyrogallol, secoisolariciresinol, sinapic acid, urolithin A, urolithin B, vanillic acid, and vanillin from Sigma-Aldrich (Steinhemin, Germany); 3-hydroxybenzoic acid, (−)-epicatechin, ferulic acid, *p*-coumaric acid, and syringic acid from Fluka (St. Louis, MO, USA); 3-(4-hydroxy-3-methoxyphenyl) propionic acid (also known as dihydroferulic acid) from Alfa Aesar (Haverhill, MA, USA); and methyl gallate from Phytolab (Vestenbergsgreuth, Germany). Acetonitrile, formic acid, and methanol were purchased from Sigma-Aldrich (Steinhemin, Germany). All other chemicals were provided by Sigma-Aldrich (Steinhemin, Germany).

2.2. Castanea sativa shells

CS were generously offered by Sortegel located in Sortes, Bragança, Portugal (latitude 41°42′18.6″N and longitude 6°48′36.6″W) in October 2018. After dehydration at 40 °C for 24 h (Excalibur Food Dehydrator, Sacramento, CA, USA), the shells were ground to 1 mm particle size using an ultra-centrifugal grinder (Retsch model ZM200, Düsseldorf, Germany) and stored in sealed flasks at room temperature in the dark.

2.3. Preparation of chestnut shells extract by supercritical fluid extraction

SFE was performed according to Pinto et al. (2020) using a supercritical fluid extractor (Waters Prep SFE System SFE-100, Milford, MA, USA) containing automated back pressure regulator, CO_2 and co-solvent pumps, heating exchangers for low and high pressures, and pressurized extraction and collection vessels. An Accel 500 LC chiller (Thermo Scientific™, Leicestershire, UK) was attached to maintain CO_2 in the liquid state. The extraction vessel was filled with a three-layer sandwich containing: Ottawa sand (5 g) in the first layer, a blend of powdered shells (15 g) and Ottawa sand (30 g) in the second layer, and Ottawa sand (5 g) in the third layer. To avoid sample projections, wool glass (Sigma-Aldrich, Steinheim, Germany) was added at the top and bottom of the extraction cell. CO_2 and ethanol supplied by Air Product and Chemicals (Allentown, PA, USA) and VWR chemicals (Radnor, PA, USA) were used, respectively, as supercritical fluid and co-solvent. The extraction conditions were 60 °C temperature, 350 bar pressure, and 15% co-solvent, based on the optimization study performed by Pinto et al. (2020). The extraction was conducted for 90 min at a constant flow rate (30 g/min). Afterward, the extract was evaporated (Rotavapor Buchi model R210, Flawil, Switzerland) and kept at −20 °C in the dark until further analysis. Three batches of extract were prepared.

2.4. In-vitro simulated gastrointestinal digestion

In-vitro simulated digestion was performed following the method implemented by Minekus et al. (2014), with slight alterations (Pinto, Silva, et al., 2023). For the gastric phase, the CS extract dissolved in distilled water (50 mg/mL) was mixed with simulated gastric fluid in a 1:1 (*v/v*) ratio and pepsin (2000 U/mL). The pH was adjusted to 3 and the mixture was incubated in a water bath (Ovan, BSC127-E model, Barcelona, Spain) at 37 °C for 2 min under stirring. Finally, the intestinal

phase was performed by mixing the gastric digest with simulated intestinal fluid in a 1:1 (*v/v*) ratio, pancreatin (100 U/mL) and bile salts (10 mM). The pH was adjusted to 7 and the mixture was incubated in a water bath at 37 °C for 2 h under stirring. After each digestion phase, aliquots were collected, centrifuged at 10,000 *g* for 10 min (Sigma 3-30KS, Osterode am HarzSigma, Germany), and kept at − 80 °C until further analysis. The simulated fluids were prepared using KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$ and $(NH_4)_2CO_3$ (Minekus et al., 2014). Three independent experiments were carried out for each digestion stage. The phenolic recovery rate was determined by applying the equation (1):

$$\text{Recovery } (\%) = (PC_{\text{digested fraction}} / PC_{\text{undigested extract}}) \times 100 \quad (1)$$

where the $PC_{\text{digested fraction}}$ is the phenolic content determined after each digestion phase, while the $PC_{\text{undigested extract}}$ is the phenolic content determined in the undigested extract. The bioaccessibility (%) refers to the recovery rate after all digestion phases.

2.5. Total phenolic and flavonoid contents

TPC and TFC were estimated by the Folin–Ciocalteu and aluminum chloride assays, respectively, using the methods previously reported by (Singleton et al., 1965) and Zhishen et al. (1999), with minor modifications (Pinto, Moreira, Švarc-Gajić, et al., 2023). The TPC results were presented in μg of gallic acid equivalents (GAE) per mg of dry weight (DW) (μg GAE/mg DW). The TFC results were expressed as μg of catechin equivalents (CE) per mg of DW (μg CE/mg DW).

2.6. In-vitro antioxidant/antiradical properties

The antioxidant/antiradical activities of undigested and digested samples were assessed by Ferric Reducing Antioxidant Power (FRAP), 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals scavenging assays, according to the procedures applied by Benzie and Strain (1996), Re et al. (1999), and Hatano et al. (1988), respectively, with minor changes (Pinto, Moreira, Vieira et al., 2023). FRAP results were expressed as μg of ferrous sulfate equivalents (FSE) per mg of DW (μg FSE/mg DW). The ABTS and DPPH results were presented, respectively, in μg of ascorbic acid equivalents (AAE) per mg of DW (μg AAE/mg DW) and μg of Trolox equivalents (TE) per mg of DW (μg TE/mg DW).

2.7. Scavenging activity against reactive oxygen and nitrogen species

The scavenging capacity of digested and undigested samples was evaluated following the procedures described by Gomes et al. (2007), with slight modifications (Pinto, Moreira, Švarc-Gajić, et al., 2023), against ROS and RNS, including superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxyl radical ($ROO^{\bullet}$), and peroxynitrite ($ONOO^-$) in the presence and absence of 25 mM $NaHCO_3$ to simulate physiological CO_2 conditions. A Synergy HT Microplate Reader (BioTek Instruments, Winooski, VT, USA) was used for absorbance and fluorescence measurements. Gallic acid and catechin were employed as positive controls. The undigested extract was tested up to 500 μg/mL. The results were expressed as inhibition, in % or half-maximal inhibitory concentration (IC_{50}, μg/mL), apart from the $ROO^{\bullet}$ scavenging assay whose results were presented as μmol of TE per mg of DW (μmol TE/mg DW).

2.8. Antioxidant enzymes activities and lipid peroxidation

The antioxidant enzymes' activities, namely catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD), were assessed using commercial enzymatic kits (Sigma-Aldrich, Steinheim, Germany). LPO was screened by determining the malondialdehyde (MDA) concentrations using a commercial kit (Merck, Darmstadt, Germany).

2.9. In-vitro biological activities

2.9.1. Acetylcholinesterase activity inhibition

AChE activity was evaluated after exposure to the undigested extract and its digests. A commercial kit (Sigma-Aldrich, St. Louis, MO, USA) estimated the amount of the colorimetric product formed by the reaction between 5,5′-dithiobis(2-nitrobenzoic acid) and thiocholine. The absorbance was measured at 412 nm. The results were presented in inhibition percentage (%).

2.9.2. Amylase activity inhibition

Amylase activity was evaluated after exposure to the undigested extract and its digests using a commercial kit (Sigma-Aldrich, St. Louis, MO, USA) that estimates the amount of the colorimetric product formed by the cleavage of ethylidene-pNP-G7 (substrate) by amylase. Nitrophenol was the standard used. The absorbance was measured at 405 nm. The results were presented in inhibition percentage (%).

2.10. Targeted metabolomic profile by LC-ESI-LTQ-Orbitrap-MS

The metabolic profile was analyzed using a LC-ESI-LTQ-Orbitrap-MS consisting of Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) with a quaternary pump, a photodiode array detector and a thermostated autosampler coupled to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) attached to an electrospray ionization (ESI) source operated in negative mode. A full scan mode was employed with a resolving power of 30,000 at *m/z* 600 and data-dependent MS/MS events were acquired with a resolving power of 15,000. Fourier transform mass spectrometry (FTMS) mode-triggered data-dependent scanning was applied to detect the most intense ions. MSn mode was applied to the ions not intense enough for a data-dependent scan. Precursors were fragmented by collision-induced dissociation using a C-trap with normalized collision energy (35 V) and an activation time of 10 ms. In FTMS mode, the mass range was *m/z* 100 to 600. An Acquity™ UPLC® BEH C18 Column (2.1 × 100 mm, i.d., 1.7 μm particle size) (Waters Corporation, Wexford, Ireland) was used for the chromatographic elution. Water (A) and acetonitrile (B) both containing 0.1% formic acid were the mobile phases used. The solvent gradient (*v/v*) of B (t (min), %B) was defined as follows: (0, 0); (2, 0); (3, 30); (4, 100); (5, 100); (6, 0); (9, 0). The column temperature was 30 °C. The injection volume was 5 μL and the flow rate was 0.450 mL/min.

The instrumental conditions were defined as described in previous studies (Pinto, Almeida, et al., 2023; Pinto, López-Yerena, et al., 2023). Phenolic compounds were identified using commercial standards, while metabolites were identified considering chemical composition, elution time, MS/MS fragmentation, and comparison with similar compounds. The remaining molecules (whose standards were not available) were identified in comparison with data published by our research group (Pinto, Almeida, et al., 2023; Pinto, López-Yerena, et al., 2023) considering retention times, chemical formula, and MS fragments, and confirmed with databases, namely food database (https://foodb.ca) and phenol database (https://phenol-explorer.eu). Identical procedures were followed in previous studies for targeted metabolic profiling in foods and extracts (Pinto, Almeida, et al., 2023; Pinto, López-Yerena, et al., 2023). MS^n measurements were performed to collect fragment ions produced in the linear ion trap. For the elemental composition of the metabolites, accurate masses, and isotopic patterns were considered. The quantification was performed using calibration curves (concentration range = 0.1–3 μg/mL, $R^2 > 0.994$) with ellagic acid, gallic acid, methyl gallate, protocatechuic acid, and pyrogallol that were previously identified as main phenolic compounds in CS (Ferreira et al., 2022; Pinto, Silva, et al., 2021; Pinto, Vieira, et al., 2021; Lameirão et al., 2020; Pinto et al., 2020). Semi-quantification was performed for the

remaining phenolic compounds and metabolites. XCalibur 3.0 software (ThermoFisher Scientific, Hemel Hempstead, UK) was used for system control and data processing. The peak area of the parent molecules was used for calculations and the results were expressed as μg of each phenolic compound equivalents per mg of DW.

2.11. Statistical analysis

Three independent experiments were performed for each assay. One-way ANOVA and Tukey's HSD test were applied for statistical analysis using IBM SPSS Statistics 24.0 software (Chicago, IL, USA). A $p < 0.05$ was established for significant differences. Multivariate data statistics were also performed by principal component analysis (PCA) and heatmap correlations using GraphPad Prism v9 software (La Jolla, CA, USA).

3. Results and discussion

3.1. Effects of gastrointestinal digestion in total phenolic and flavonoid contents

The richness of CS in phenolic compounds and flavonoids has been demonstrated by previous studies, exploring the efficacy of different extraction techniques and conditions (Pinto, Silva, et al., 2021; Pinto, Vieira, et al., 2021; Lameirão et al., 2020; Pinto et al., 2020). Nonetheless, the effects of *in-vitro* gastrointestinal digestion on the phenolic composition of CS extract have not been explored. The TPC and TFC of CS extract prepared by SFE were determined before and after *in-vitro* simulated digestion (Table 1).

The results showed significant differences in the TPC and TFC results after *in-vitro* digestion of CS extract, emphasizing the substantial impact of gastric and intestinal phases in the phenolic and flavonoid concentrations. Similar TPC was estimated for CS extract after gastric and intestinal digestion (37.96 and 38.57 μg GAE/mg DW, respectively), revealing non-significant differences ($p > 0.05$). Oppositely, the undigested extract (127.72 μg GAE/mg DW) had a significantly ($p < 0.05$) higher TPC when compared to its digests. The present results indicate that gastric conditions (i.e., pepsin and acidic pH) allow the release of most the phenolic compounds from the extract to the digestion medium, remaining after intestinal digestion (highlighting their stability under intestinal conditions) or being metabolized into other phenolic compounds that become potentially available for intestinal absorption.

The TFC of the CS extract also significantly decreased ($p < 0.05$) after *in-vitro* digestion, following this order: undigested extract (54.15 μg CE/mg DW) > intestinal digest (15.03 μg CE/mg DW) > gastric digest (10.65 μg CE/mg DW). From gastric to intestinal digestion, the TFC increased by 41%. In contrast to TPC, flavonoids were recovered more effectively under intestinal environment, highlighting that the action of pancreatin, bile salts and neutral pH enhanced the flavonoids release from the extract to the digestive medium, which agrees with recent studies on cupuassu peels, blueberry fruits, roasted coffee beans, and functional cookies enriched with phenolic extracts (Andrade et al., 2022; Muñoz-Fariña et al., 2023; Pinto, Moreira, Švarc-Gajić et al., 2023; Wu et al., 2022). This explanation is corroborated by the significantly different results ($p < 0.05$) between the gastric and intestinal phases.

The decrease in TPC and TFC after the *in-vitro* gastrointestinal digestion was already expected, as proven in previous studies for different food by-products (Andrade et al., 2022; Kashyap et al., 2022; Ruíz-García et al., 2022; Tu et al., 2021). This may be explained by the phenolic compounds' instability to digestive enzymes and pH changes that lead to their degradation or biotransformation into metabolites. Another hypothesis could be associated to possible interactions with other constituents of the digestive media or extract (e.g., amino acids, fatty acids, sugar moieties) that may form complexes with some phenolics and interfere with the TPC quantification (Hu et al., 2023). Recent studies have reported lower TPCs after *in-vitro* digestion, particularly for cherry pomace, cupuassu seeds, and grape skins (Andrade et al., 2022; Kashyap et al., 2022; Ruíz-García et al., 2022), which may be explained by the low solubility of phenolic compounds in the digestive media or their metabolization into compounds resulting from interactions with other constituents from the digestive media (e.g., amino acids) (Hu et al., 2023). Recently, Tu et al. (2021) investigated the bioaccessibility of phenolic compounds from Chinese chestnut (*Castanea molissima*) shells and examined their metabolic transformations upon *in-vitro* simulated digestion. The results were in line with the current study, confirming a decrease in TPC during gastrointestinal digestion (gastric phase: 83.58 and 47.14 mg GAE/100 mL, respectively, for chestnut outer and inner shells; intestinal phase: 56.69 and 32.47 mg GAE/100 mL, respectively) (Tu et al., 2021).

Table 1
Total phenolic and flavonoid contents, bioaccessibility, and antioxidant activity of chestnut shells (CS) extract prepared by supercritical fluid extraction (SFE) before and after *in-vitro* digestion.

	Undigested CS extract	*In-vitro* simulated digestion	
		Gastric digest	Intestinal digest
TPC (μg GAE/mg DW)	127.72 ± 2.68^{a}	37.96 ± 3.43^{b}	38.57 ± 5.33^{b}
Phenolics recovery (%)	–	29.73 ± 2.80^{a}	30.25 ± 4.34^{a}
TFC (μg CE/mg DW)	54.15 ± 5.00^{a}	10.65 ± 0.63^{c}	15.03 ± 1.33^{b}
Flavonoids recovery (%)	–	19.80 ± 1.41^{b}	27.74 ± 2.03^{a}
ABTS (μg AAE/mg DW)	119.80 ± 6.37^{a}	27.15 ± 0.72^{c}	57.85 ± 0.35^{b}
DPPH (μg TE/mg DW)	73.33 ± 2.95^{a}	33.20 ± 2.86^{c}	49.44 ± 3.97^{b}
FRAP (μg FSE/mg DW)	318.05 ± 11.66^{a}	189.25 ± 18.49^{c}	260.12 ± 20.99^{b}

AAE, ascorbic acid equivalents. CE, catechin equivalents. CS, chestnut shells. DW, dry weight. FSE, ferrous sulfate equivalents. FRAP, ferric reducing antioxidant power. GAE, gallic acid equivalents. TE, Trolox equivalents. TFC, total flavonoid content. TPC, total phenolic content. Different letters (a, b, and c) denote significant differences among samples ($p < 0.05$).

3.2. Bioaccessibility of phenolic compounds

Bioaccessibility corresponds to the concentration of phenolic compounds released from the extract after gastrointestinal digestion, becoming potentially available for intestinal absorption (Pinto, Silva et al., 2023). The phenolic recovery rates were 29.73% and 30.25%, respectively, after gastric and intestinal digestion of CS extract (Table 1). These results were identical ($p > 0.05$), emphasizing the hypothesis that most of the phenolic compounds present in the extract were recovered in the gastric environment (i.e., acidic pH and pepsin), while a similar fraction of these compounds was also retained in the intestinal medium after the action of pancreatin, bile salts and neutral pH (Pinto, Moreira, Švarc-Gajić et al., 2023).

The flavonoid recovery rates showed a distinct behavior, increasing significantly ($p < 0.05$) from gastric (19.80%) to intestinal (27.74%) digestion. Although a substantial portion was retained under gastric conditions, flavonoids were released mostly by gut enzymes and under neutral pH.

The bioaccessibility of phenolic and flavonoid compounds indicated a satisfactory release profile of the CS extract, with a maximum bioaccessibility of 30% and following the same trends of TPC and TFC. These findings corroborated the TPC and TFC results, indicating that phenolic compounds and flavonoids become more bioaccessible upon *in-vitro* digestion due to the slight increase in their recovery rates. This trend was also attested for other fruit by-products after *in-vitro* digestion, such as grape skin and cherry pomace, reporting bioaccessibility rates of up to 50% (Kashyap et al., 2022; Ruíz-García et al., 2022). Recent studies have implemented encapsulation techniques to improve the bioaccessibility of phenolic compounds recovered from fruit by-

products, such as acerola residue and pomegranate peel (Andishmand et al., 2023; Silva et al., 2022).

The recovery rates may be justified by the interaction of phenolic compounds and flavonoids with other CS extract components, such as minerals, pigments, fatty acids, and sugars, that can be sensitively activated by the acidic and alkaline environment (Hu et al., 2023). Thus, phenolic compounds could precipitate with other substances present in the digests under acidic and alkaline pH (Hu et al., 2023; Pinto, Silva et al., 2023). Furthermore, it is important to highlight that phenolic compounds not released in gastric and intestinal digestions may be retained in the indigestible fraction and be further biotransformed in colon, releasing aglycones and microbial metabolites with interesting bioactive properties (Pinto, Almeida, et al., 2023; Pinto, López-Yerena, et al., 2023).

3.3. Effects of gastrointestinal digestion on antioxidant/antiradical properties

Natural antioxidants from CS have been documented for food and nutraceutical applications due to their disease-preventive properties on chronic diseases mediated by oxidative stress (e.g., cancer, diabetes, premature aging, neurological, cardiovascular, and metabolic pathologies) as well as food preservation properties (replacing synthetic preservatives to extend shelf-life, and preventing lipid oxidation and microorganisms' proliferation) (Pinto, Cádiz-Gurrea, Vallverdú-Queralt et al., 2021). Different studies have provided *in-vitro* and *in-vivo* evidence of the disease-preventing properties of CS, including against oxidative stress-triggered diseases, type 2 diabetes, microbial infections, and gastritis (Pinto, Almeida, et al., 2023; Pinto, Ferreira, et al., 2023; Pinto, López-Yerena, et al., 2023; Sangiovanni et al., 2018; Tsujita et al., 2008; Pinto, Silva, et al., 2021; Pinto et al., 2023). The use of CS extract as a functional ingredient requires the assessment of its bioactivity after digestion. To the best of our knowledge, this is the first study that evaluates the impact of digestion on the antioxidant/antiradical properties of CS extract prepared by SFE (Table 1).

As shown in Table 1, the antioxidant/antiradical properties of the CS extract increased in the following order: gastric digest < intestinal digest < undigested extract, highlighting a better antioxidant response for the undigested extract, along with an improvement in the antioxidant effects during *in-vitro* digestion. The three antioxidant assays revealed similar patterns.

Regarding antiradical activity, the ABTS response was twice as high after intestinal digestion (57.85 μg AAE/mg DW) when compared to the gastric phase (27.15 μg AAE/mg DW), with significant differences ($p < 0.05$) between the two phases. Compared with the undigested extract (119.80 μg AAE/mg DW), the antiradical activity after gastric and intestinal digestion was, respectively, 4.4-fold and 2.1-fold lower. An identical response was observed in the DPPH radicals scavenging assay, with a 49% increase from gastric to intestinal digestion, achieving significant differences ($p < 0.05$). The antiradical potential was 2.2 and 1.5 times lower, respectively, after the gastric and intestinal phases (33.20 and 49.44 μg TE/mg DW, respectively), when compared to the undigested extract (73.33 μg TE/mg DW). Additionally, significantly different antiradical responses ($p < 0.05$) were observed between the two digestive phases and the undigested extract in the ABTS and DPPH assays.

The antioxidant activity assessed by the FRAP assay also increased 37% from gastric to intestinal digestion, with significant differences ($p < 0.05$) between digests. Compared with the undigested extract (318.05 μg FSE/mg DW), the antioxidant potential was 68% and 22% lower, respectively, after gastric and intestinal digestion (189.25 and 260.12 μg FSE/mg DW, respectively). The FRAP responses were significantly different ($p < 0.05$) between gastric and intestinal digests and the undigested extract.

Overall, the undigested CS extract revealed better antioxidant/antiradical properties, which corroborates recent studies on the digestibility of antioxidants from food by-products (Andrade et al., 2022; Muñoz-Fariña et al., 2023; Wu et al., 2022). Considering only the digests, better antioxidant/antiradical effects were obtained after intestinal digestion, proposing that higher concentrations of antioxidants were recovered at this phase or that the bioactive molecules retrieved had better antioxidant/antiradical properties (Pinto, Moreira, Švarc-Gajić et al., 2023).

In general, antioxidant/antiradical results are in close agreement with TPC and TFC, reinforcing the promising role of phenolic and flavonoid compounds in the bioactivity of undigested and digested CS extract. The hydroxyl groups released from phenolic monomers or aglycones in gastric digestion that provide effective antioxidant properties may be a plausible explanation for the mild antioxidant properties after gastric digestion (Wu et al., 2022). Furthermore, phenolic compounds are biotransformed into small molecules recognized as metabolites and endowed with potent antioxidant properties under intestinal conditions (by the action of gut enzymes and neutral pH), explaining the more pronounced increase in antioxidant/antiradical responses after intestinal digestion (Muñoz-Fariña et al., 2023).

According to Tu et al. (2021), lower antioxidant results were reported for Chinese CS before (50.27 and 50.85 mg AAE/100 mL, respectively, for chestnut outer and inner shells in DPPH assay) and after gastric (99.71 and 74.79 mg AAE/100 mL, respectively) and intestinal (90 and 70 mg AAE/100 mL, respectively) digestion. Previous reports have shown similar patterns with an increase in antioxidant/antiradical properties of phenolic extracts during *in-vitro* digestion (Muñoz-Fariña et al., 2023; Pinto, Silva, et al., 2023).

The antioxidant properties of the CS extract digested fractions may be due to the phenolic compounds released from the extract to the digestive media, including free phenolics and soluble-bound phenolics (e.g., esterified) that are linked to sugars through a hydroxyl group or carbon–carbon linkages (Hu et al., 2023). Additionally, other soluble molecules with antioxidant properties (e.g., minerals, vitamins, fatty acids, fibers) may be present in the CS extract and could be released to the digestive media, affecting the ability to scavenge free radicals (in ABTS and DPPH assays) and reduce ferric ions (in FRAP assay) (Andrade et al., 2022; Hu et al., 2023; Wu et al., 2022). The differences observed in the antioxidant/antiradical results may be due to the different methodologies principles as well as the bioaccessibility of the phenolic compounds since the chemical structures are directly related to the antioxidant efficiency.

3.4. Scavenging efficiency against reactive oxygen and nitrogen species

Natural antioxidants have been described as promising anti-aging molecules with potential uses as nutraceuticals or functional ingredients for foods. CS are excellent sources of phenolic compounds and vitamin E that deliver protective effects against oxidative stress and inflammation-induced injuries in biomolecules (Pinto, Almeida et al., 2023; into, Cádiz-Gurrea, Vallverdú-Queralt, et al., 2021). The results of scavenging assays against ROS and RNS produced in the human body before and after *in-vitro* digestion of CS extract are presented in Supplementary Table 1.

The highest scavenging efficiency was achieved against HOCl and $ONOO^-$ in the presence and absence of sodium bicarbonate. The scavenging capacity of the samples improved as follows: gastric digest < intestinal digest < undigested extract, except for $O_2^{\bullet-}$ and $ROO^{\bullet}$ in which the digests showed better results when compared to the undigested extract.

Considering $O_2^{\bullet-}$ scavenging potential, the CS extract showed promising results after gastric and intestinal digestion (55.71 and 57.41% inhibition, respectively), while the undigested extract inhibited 49.42% of the $O_2^{\bullet-}$ generated. Among the positive controls, catechin reached a 4.4-fold higher IC_{50} value (48.21 μg/mL) when compared to gallic acid (10.95 μg/mL). Significant differences ($p < 0.05$) were achieved between undigested extract and its digests, while non-significant differences ($p > 0.05$) were observed between gastric and intestinal

digests.

Regarding the H_2O_2 quenching assay, the undigested extract (69.97% inhibition) and its intestinal digest (59.78% inhibition) scavenged this species more efficiently, followed by gastric digest (50.38% inhibition). However, the positive controls tested showed significantly higher H_2O_2 scavenging ability. Catechin displayed a 5-fold lower IC_{50} value (20.78 μg/mL) when compared to gallic acid (106.03 μg/mL). Additionally, the results of the undigested extract and its digests were significantly different ($p < 0.05$).

In what concerns to the HOCl quenching assay, the best scavengers were catechin ($IC_{50} = 0.37$ μg/mL) and undigested CS extract ($IC_{50} = 1.57$ μg/mL). Among the digested samples, the intestinal digest (75.37% inhibition) achieved significantly ($p < 0.05$) higher efficiency when compared to the gastric digest (71.19% inhibition).

The $ROO^{\bullet}$ scavenging potential was estimated through the oxygen radical absorbance capacity (ORAC) assay to assess the ability of undigested and digested CS extract to protect biological tissues against LPO (Pinto et al., 2021). The undigested CS extract and its gastric and intestinal digests achieved identical $ROO^{\bullet}$ scavenging responses ($p > 0.05$), with results ranging between 0.05 and 0.07 μmol TE/mg DW. Catechin (1.84 μmol TE/mg DW) and gallic acid (1.09 μmol TE/mg DW) showed the highest $ROO^{\bullet}$ quenching ability.

The $ONOO^-$ scavenging activity assay was performed in the absence and presence of sodium bicarbonate to simulate physiological bicarbonate concentrations (≈25 mM) (Pinto et al., 2021). Among samples, $ONOO^-$ counteracting responses improved in the following order: gastric digest < intestinal digest < undigested extract, with better results in the presence of sodium bicarbonate (64.85% inhibition, 71.09% inhibition, and IC_{50} of 3.20 μg/mL, respectively) than in its absence (62.28% inhibition, 70.15% inhibition, and IC_{50} of 3.80 μg/mL). Nonetheless, catechin and gallic acid were better $ONOO^-$ quenchers in the presence of sodium bicarbonate (IC_{50} of 0.23 and 0.29 μg/mL, respectively) as well as in its absence (IC_{50} of 0.16 and 0.15 μg/mL). The undigested extract and its digests showed significantly different results ($p < 0.05$), while catechin and gallic acid revealed similar ($p > 0.05$) scavenging efficiency.

The promising results observed were probably attributed to the phenolic composition of the CS extract and respective digests, namely phenolic acids, flavonoids, hydrolyzable tannins, and lignans (identified in this study by metabolomic analysis), whose scavenging potential has been previously reported (Ketsawatsakul et al., 2000; Pinto, Ferreira, et al., 2023). These outcomes are in line with the ones reported for CS extracts prepared by other eco-friendly technologies (Pinto, Silva, et al., 2021; Pinto, Vieira, et al., 2021; Lameirão et al., 2020; Pinto et al., 2020).

3.5. Antioxidant enzymes activities and lipid peroxidation

Antioxidant enzymes protect the human body against the deleterious effects of pro-oxidant species, minimizing the impact of oxidative stress on biomolecules (Pinto, Reis et al., 2021). However, their activities decrease with age and poor diet (Pinto, Reis et al., 2021). In this sense, antioxidants-rich nutraceuticals may offer a valuable ally against oxidative stress, enhancing the activity of these antioxidant enzymes

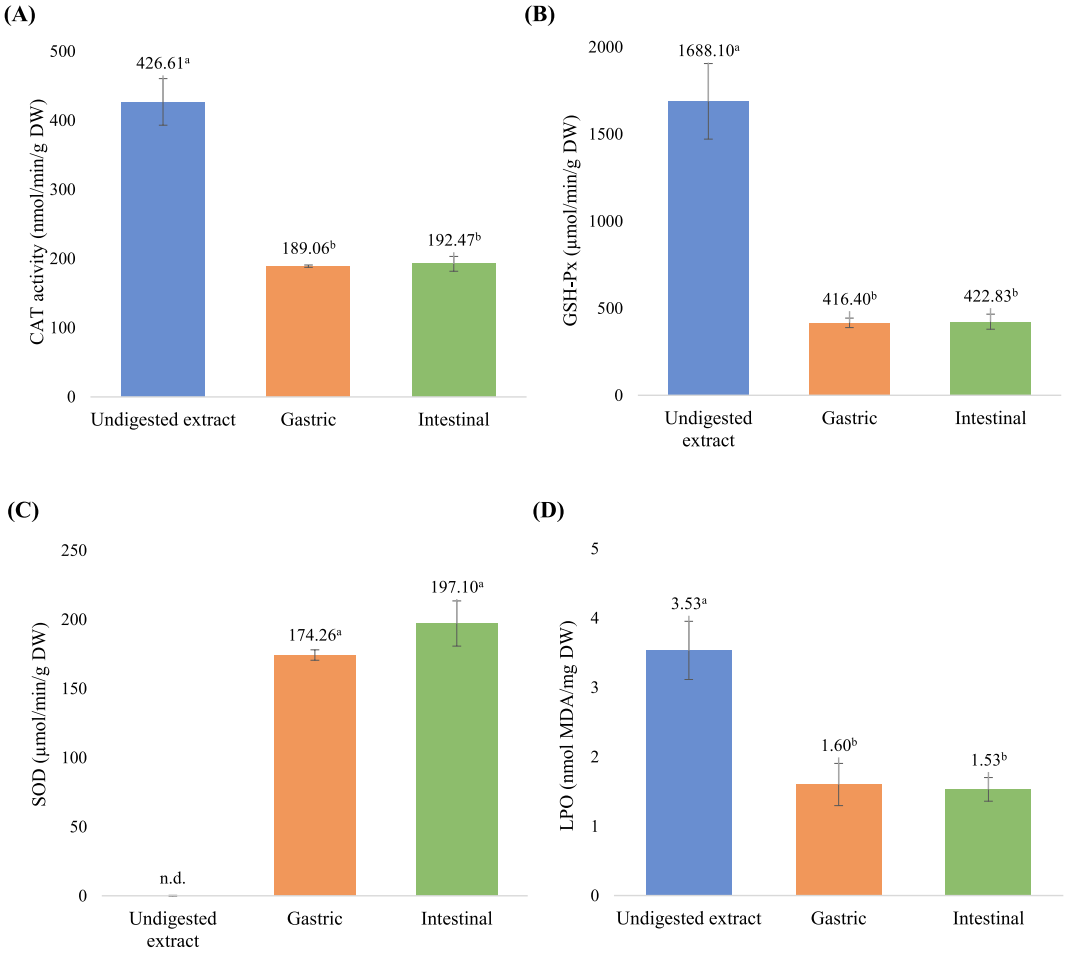

Fig. 1. Antioxidant enzymes activities, namely catalase (A), glutathione peroxidase (B) and superoxide dismutase (C), and lipid peroxidation (D) of undigested and digested chestnut shells (CS) extract prepared by supercritical fluid extraction (SFE). Different letters (a and b) denote significant differences ($p < 0.05$) between samples.

(Pinto, Almeida, et al., 2023; Pinto, Cádiz-Gurrea, Vallverdú-Queralt, et al., 2021). The effects of undigested CS extract and its digests on antioxidant enzymes' activities and LPO are shown in Fig. 1.

The present results indicate an improvement in the activities of antioxidant enzymes, namely CAT, SOD, and GSH-Px, and a protective effect against LPO. These findings highlight the antioxidant properties of CS extract before and after *in-vitro* digestion, corroborating previous results.

Considering the CAT activity (Fig. 1A), the undigested CS extract (426.61 nmol/min/g DW) revealed a 2.2-fold higher result when compared to its digests, while the gastric (189.06 nmol/min/g DW) and intestinal (192.47 nmol/min/g DW) digests achieved similar capacities ($p > 0.05$) to improve the CAT activity. Additionally, significant differences ($p < 0.05$) were observed between the undigested extract and its digests.

An identical response was observed for GSH-Px activity (Fig. 1B). Gastric and intestinal digests of the CS extract revealed similar capacities to improve GSH-Px activity (416.40 and 422.83 μmol/min/g DW, respectively), without significant differences ($p > 0.05$). Furthermore, CS extract stimulated 4 times more the GSH-Px activity before *in-vitro* digestion (1688.10 μmol/min/g DW). The result of the undigested extract was significantly different ($p < 0.05$) from its gastric and intestinal digests.

The SOD activity increased in the presence of gastric and intestinal digests (174.26 and 197.10 μmol/min/g DW, respectively), without significant differences ($p > 0.05$) (Fig. 1C). In contrast to the CAT and GSH-Px assays, the undigested extract showed no effect on SOD activity.

The LPO is an effective marker of oxidative damage, playing a central role in the pathogenesis of chronic diseases. The CS extract after gastric and intestinal digestion prevented efficiently the LPO-induced injuries, disclosing the lowest results (1.60 and 1.53 nmol MDA/mg DW, respectively) (Fig. 1D). No significant differences ($p > 0.05$) were observed between gastric and intestinal digests, highlighting similar effects on LPO. A lower protective response was obtained for the undigested extract (3.53 nmol MDA/mg DW), with significant differences ($p < 0.05$) when compared to both digests.

Overall, the CS extract improved the activities of antioxidant enzymes and prevented LPO before and after *in-vitro* digestion. These results suggest a slow release of antioxidant molecules from the extract during digestion, probably retaining a significant concentration of antioxidants after the gastric and intestinal phases, which explains the antioxidant properties of these digests. Moreover, these results are in agreement with TPC, TFC and antioxidant/antiradical results previously discussed, supporting that phenolic compounds may be effective molecules in the activation of antioxidant enzymes as well as in the prevention of LPO, as suggested by other authors (Gawlik-Dziki, 2014; Pinto, Almeida, et al., 2023). In a recent study, Pinto, Almeida, et al. (2023) demonstrated an improvement in the activities of antioxidant enzymes along with a protective response against LPO in the blood serum, liver, and kidney from rats treated with 50 and 100 mg/kg body weight of CS extract prepared by subcritical water extraction. Likewise, Gawlik-Dziki (2014) proved the activation of antioxidant enzymes induced by different spice extracts, including basil, ginger, marjoram, pepper, and thyme. Ginger, marjoram, and thyme extracts stimulated the SOD activity after *in-vitro* digestion, with better results after the intestinal phase (<1 mg/g DW of extract required to induce 50% activation of SOD activity), while a similar trend was observed in the CAT response after exposure to the marjoram extract, with a 50% decrease in the inhibitory activity after intestinal digestion when compared to the undigested extract (Gawlik-Dziki, 2014).

3.6. Inhibition of acetylcholinesterase and α-amylase activities

Besides antioxidant properties, phenolics-rich extracts have been explored as promising nutraceutical ingredients useful in the prevention and co-therapy of metabolic and neurological pathologies, due to their hypoglycemic and neuroprotective effects (Pinto, Silva, et al., 2021). To the best of our knowledge, this is the first study evaluating the hypoglycemic and neuroprotective properties of CS extract prepared by SFE after *in-vitro* digestion. The inhibition results of AChE and α-amylase activities induced by CS extract before and after *in-vitro* digestion are shown in Fig. 2.

Considering the AChE activity, the undigested CS extract reached more than 70% inhibition at a concentration of 125 μg/mL, which was significantly ($p < 0.05$) higher when compared to its digests. A 32% and 30% decrease in the AChE inhibition was obtained, respectively, after the gastric and intestinal phases. Nonetheless, the CS extract after gastric (38.47%) and intestinal (40.34%) digestion still effectively inhibited the AChE activity, revealing similar results ($p > 0.05$). These outcomes agree with the TPC, TFC, and bioaccessibility results, reinforcing the contribution of phenolic compounds and flavonoids in the extract as well as their slow release during digestion. In addition, the identical results of gastric and intestinal digests suggest that the phenolic compounds endowed with neuroprotective properties are mainly released in the gastric phase and preserved under intestinal conditions. In another study, Pinto, Silva, et al. (2021) proved the anticholinergic activity of CS extract prepared by microwave-assisted extraction (MAE), reaching up to 29.94% inhibition at 1000 μg/mL. Murugan et al. (2016) demonstrated lower inhibitory enzymatic properties in fruit and seed extracts of Algerian dates (*Phoenix loureirii*) on AChE activity before (<20% and < 40%, respectively) and after intestinal digestion (<30% and < 35%, respectively).

The undigested CS extract also induced a moderate inhibitory response on α-amylase activity, with 15.09% inhibition at 125 μg/mL. The α-amylase inhibitory potential was maintained even after *in-vitro* digestion, revealing mild hypoglycemic properties after the gastric and intestinal phases (14.12% and 12.98% inhibition, respectively). Notably, similar inhibitory effects ($p > 0.05$) were observed before and after gastric and intestinal digestion of CS extract. The hypoglycemic effects observed are possibly attributed to the phenolic and flavonoid compounds present in the CS extract. Furthermore, the identical responses on α-amylase activity before and after digestion highlight that most of the bioactive molecules in CS extract were efficiently released during digestion, exerting their pro-healthy benefits (particularly hypoglycemic properties). Pinto, Silva, et al. (2021) demonstrated the anti-amylase activity of the CS extract prepared by MAE, with inhibition percentages varying between 7.81% (at 125 μg/mL) and 15.22% (at 1000 μg/mL). Recently, Peláez-Acero et al. (2022) reported similar responses of Mexican honey from different botanical origins in α-amylase and α-glucosidase inhibition after *in-vitro* digestion. The undigested honey inhibited 15.16–37.15% of α-amylase activity, decreasing

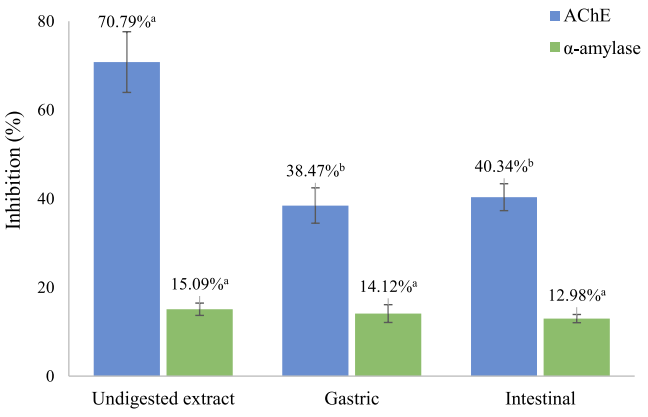

Fig. 2. Inhibition of AChE and α-amylase activities of undigested and digested chestnut shells (CS) extract prepared by supercritical fluid extraction (SFE). Different letters (a and b) denote significant differences ($p < 0.05$) between samples.

significantly after gastric digestion (9.03–19.56%) and, to a higher extent, after intestinal digestion (2.12–14.13%). The authors also attributed this bioactivity of honey extracts to their phytochemical composition rich in phenolic compounds and flavonoids (Peláez-Acero et al., 2022).

The promising inhibitory potential observed is probably ascribed to the phenolic composition of the CS extract and its digests (mainly phenolic acids, flavonoids, and hydrolyzable tannins identified in the metabolomic analysis) that demonstrated mild to strong anti-amylase and anti-AChE activities, exerting hypoglycemic and neuroprotective effects (Peláez-Acero et al., 2022; Pinto, Moreira, Švarc-Gajić, et al., 2023; Pinto, Cádiz-Gurrea, Vallverdú-Queralt, et al., 2021). The anti-AChE activity showed positive correlations with TPC ($r^2 \leq 0.42$) and TFC ($r^2 \leq 0.57$), while anti-amylase activity only revealed mild positive correlations with TPC ($r^2 \leq 0.65$), reinforcing the prevailing contribution of phenolic and flavonoid compounds for the neuroprotective and hypoglycemic properties observed in undigested and digested CS extract. Noteworthy, these results are in close agreement with the TPC and TFC values of digested and undigested CS extract.

3.7. Targeted metabolomic profile by LC-ESI-LTQ-Orbitrap-MS

During metabolism, phenolic compounds are biotransformed into metabolites, influencing their bioaccessibility and, subsequently, their bioactivity in human health (Pinto, Almeida, et al., 2023). Various *in-vitro* models have been designed to simulate human gastrointestinal digestion, aiming to evaluate the effects of metabolism on the phenolic composition and overcome the limitations of *in-vivo* assays (such as restraining the interindividual variability and interactions with other food nutrients) (Pinto, Almeida, et al., 2023; Pinto, López-Yerena, et al., 2023). Table 2 presents the phenolic compounds identified in the CS extract prepared by SFE before and after *in-vitro* digestion. Table 3 presents the concentrations of phenolic compounds in the undigested CS extract and its gastric and intestinal digests.

A total of 45 compounds were identified in the undigested extract, representing 45.78 μg/mg DW. The total phenolic concentrations decreased significantly ($p < 0.05$) after *in-vitro* digestion. Similar phenolic concentrations ($p > 0.05$) were determined after gastric (36.75 μg/mg DW) and intestinal (35.54 μg/mg DW) digestion, with recovery rates of 80.29% and 77.64%, respectively. Only 42 and 24 compounds were identified, respectively, in gastric and intestinal digests. These results highlight substantial variations in the phenolic profile of the CS extract upon *in-vitro* digestion, reinforcing the significant impact of pH and digestive enzymes on phenolic compounds retained after each digestive phase that probably suffered molecular modifications (Pinto, Moreira, Švarc-Gajić, et al., 2023).

The main polyphenolic class was phenolic acids, representing 92.8%, 97.6%, and 99.2% of the total content, respectively, in the undigested extract, and its gastric and intestinal digests. Among phenolic acids, phenylpropanoic acids (44.9%, 57.2%, and 83.7%, respectively, in undigested extract, gastric and intestinal digests), hydroxybenzoic acids (33.1%, 25.3%, and 8.0%, respectively), and phenylacetic acids (13.9%, 14.5% and 7.0%, respectively) were the most abundant subclasses. The differences in the phenolic composition of digested and undigested CS extract may be due to the presence of gastric and intestinal enzymes (such as pepsin and pancreatin) that induce changes in the original phenolic compounds, with subsequent influence on the bioaccessible fraction (Andrade et al., 2022). Furthermore, the phenolic compounds released can suffer complexation with other molecules from the CS extract or the digestive media (e.g., fatty acids, amino acids, sugars) during digestion, resulting in additive, neutralization, or synergistic interactions with bioactive compounds (Hu et al., 2023; Wu et al., 2022).

Considering hydroxybenzoic acids, gallic acid was the main compound in the undigested extract (9.17 μg/mg DW). However, the gallic acid concentration decreased by half after gastric digestion and only reached 4.08% recovery after the intestinal phase, suggesting its metabolization under gastric conditions, which originates 2,5-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, and 3-hydroxybenzoic acid (via dehydroxylation) together with catechol (via decarboxylation and dehydroxylation), explaining the recovery rates above 100% for these compounds in the gastric phase (Bento-Silva et al., 2020). Significant differences ($p < 0.05$) were observed between both digests and undigested extract. This explanation may also justify the presence of 3-hydroxybenzoic acid as the main compound in the intestinal digest. Methyl gallate may be also biotransformed into simpler hydroxybenzoic acids, such as 3-hydroxybenzoic acid (Pinto, López-Yerena, et al., 2023). However, these compounds showed low stability in intestinal conditions, as evidenced by the low recovery rates, probably being degraded or metabolized into smaller molecules by gut enzymes acting at neutral pH. Syringic acid was also quantified at higher concentrations in the gastric digest, with a recovery of 165% that may have resulted from sinapic acid metabolism or be released by hydrolysis of syringic acid-*O*-glucoside (Bento-Silva et al., 2020). According to Table 3, sinapic acid was quantified in the undigested extract at 0.1 μg/mg DW. Additionally, two sinapic acid derivatives were also identified, namely sinapic acid-*O*-glucoside and sinapoylquinic acid, which can lose glucose and quinic acid moieties, respectively, originating sinapic acid that may be further metabolized into syringic acid (which was detected in the undigested extract and respective digests) (Bento-Silva et al., 2020). Alternatively, sinapic acid may also be produced from ferulic acid (also detected in the undigested extract) by hydroxylation and subsequent *O*-methylation, and directly metabolized to syringic acid (Bento-Silva et al., 2020). Protocatechuic acid, also known as 3,4-dihydroxybenzoic acid, was not identified after *in-vitro* digestion, probably due to its bioconversion into β-ketoadipate via ring cleavage that further originates succinyl-coenzyme A (CoA) and acetyl-CoA (Alvarez-Rodríguez et al., 2003). Glycosidic and quinic acid derivatives of hydroxybenzoic acids were not identified or were only quantified at trace levels after digestion, which may be explained by the hydrolysis of these phenolic acids derivatives, releasing their aglycones, glucose, and quinic acids (Bento-Silva et al., 2020; Pinto, López-Yerena, et al., 2023). The concentrations of hydroxybenzoic acids decreased significantly ($p < 0.05$) in the following order: undigested extract (15.15 μg/mg DW) > gastric digest (9.28 μg/mg DW) > intestinal digest (2.85 μg/mg DW), with recovery rates of 61.25% and 18.83%, respectively, for gastric and intestinal digests.

Regarding hydroxycinnamic acids, low concentrations were determined in the undigested extract and its digested fractions (up to 0.43 μg/mg DW). Caffeic acid, chlorogenic acid, ferulic acid, and *p*-coumaric acid were the only hydroxycinnamic acids identified and quantified in the intestinal digest. Furthermore, caffeic acid-*O*-glucoside and sinapoylquinic acid were identified, but not quantified in the gastric digest. Sinapic acid was quantified in higher concentrations in the gastric digest than in the undigested extract, revealing a recovery rate above 100% that can be justified by the hydrolysis of sinapoylquinic acid and sinapic acid-*O*-glucoside (present in the undigested extract) into sinapic acid, quinic acid and glucose by glycosidases under acidic pH of the stomach (Sova & Saso, 2020). The same applies to *p*-coumaric acid with recovery rates above 100% in both digests that may derived from coumaroylquinic acid and coumaric acid-*O*-glucoside (Sova & Saso, 2020). Glycosidic derivatives of caffeic, coumaric, ferulic, and sinapic acids, as well as quinic acid derivatives of coumaric, ferulic, and sinapic acids, were not identified after intestinal digestion, possibly due to their hydrolysis into aglycones, glucose and quinic acid (Pinto, López-Yerena, et al., 2023; Sova & Saso, 2020). This may explain the almost 100% recovery rates of caffeic, *p*-coumaric, ferulic, and sinapic acids after gastric and intestinal digestion. Although quinic acid was not identified due to the lack of available standard, different studies performed in similar matrices identified quinic acid as a metabolite resulting from quinic acids derivatives of hydroxybenzoic and hydroxycinnamic acids (Bento-Silva et al., 2020; Pinto, Ferreira, et al., 2023; Pinto, López-Yerena, et al., 2023; Sova & Saso, 2020). Nevertheless, the identification

Table 2
Identification of phenolic compounds and their metabolites in chestnut shells (CS) extract prepared by supercritical fluid extraction (SFE) before and after *in-vitro* digestion explored by LC-ESI-LTQ-Orbitrap-MS.

Compound	Neutral molecular formula	Rt (min)	Ion mass [M−H]⁻ Theoretical	Experimental	Error (amu)	MS^2 fragment ions [M−H]⁻	*In-vitro* digestion Gastric	Intestinal	Undigested CS extract
Gallic acid	$C_7H_6O_5$	0.70	169.0142	169.0139	0.7600	125.02402	+	+	+
Monogalloyl glucose	$C_{13}H_{16}O_{10}$	0.79	331.0665	331.0653	−0.6928	169.01402, 241.03403, 271.04417	−	−	+
Galloylquinic acid	$C_{14}H_{16}O_{10}$	0.87	343.0665	343.0649	−1.0632	169.01398, 191.05526	−	−	+
Galloylshikimic acid	$C_{14}H_{14}O_9$	1.10	325.0560	325.0543	−1.1277	125.02410, 169.01404	−	−	+
Pyrogallol	$C_6H_6O_3$	2.20	125.0244	125.0240	0.6590	81.03398, 97.02871	+	+	+
Protocatechuic acid	$C_7H_6O_4$	4.10	153.0193	153.0190	0.7351	109.02913, 125.02402	−	−	+
3,5-Dihydroxybenzoic acid	$C_7H_6O_4$	4.45	153.0193	153.0192	0.9151	109.02910	+	+	+
Protocatechuic acid-*O*-glucoside	$C_{13}H_{16}O_9$	4.61	315.0722	315.0702	−0.8775	109.02865, 153.01896	−	−	+
Hydroxybenzoic acid-*O*-glucoside	$C_{13}H_{16}O_8$	4.64	299.0772	299.0756	−0.5435	137.02423	+	−	+
Catechol	$C_6H_6O_2$	4.68	109.0295	109.0290	0.5638	66.08643	+	+	+
Syringic acid-*O*-glucoside	$C_{15}H_{20}O_{10}$	4.74	359.0983	359.0966	−0.7130	197.04483	+	−	+
Sinapic acid-*O*-glucoside	$C_{17}H_{22}O_{10}$	4.75	385.1140	385.1119	−1.0032	223.06018	+	−	+
Chlorogenic acid	$C_{16}H_{18}O_9$	4.79	353.0878	353.0860	−0.6794	191.03434	+	+	+
4-Hydroxybenzoic acid	$C_7H_6O_3$	4.80	137.0244	137.0241	0.7491	93.03388	+	+	+
Hydroxyhippuric acid	$C_9H_9NO_4$	4.82	194.0453	194.0449	0.1256	176.06048	+	+	−
2,5-Dihydroxybenzoic acid	$C_7H_6O_4$	4.85	153.0193	153.0189	0.6448	109.02892	+	−	+
Methyl gallate	$C_8H_8O_5$	4.86	183.0299	183.0294	0.5800	125.02809, 169.01788	+	+	+
Dihydroxyphenylpropionic acid	$C_9H_{10}O_4$	4.87	181.0506	181.0504	0.8254	137.06011	+	+	−
(*Epi*)catechin	$C_{15}H_{14}O_6$	4.90	289.0718	289.0702	−0.4752	245.08250	+	+	+
3-Hydroxybenzoic acid	$C_7H_6O_3$	4.91	137.0244	137.0238	0.4392	93.03386	+	+	+
Caffeic acid	$C_9H_8O_4$	4.92	179.0349	179.0345	0.6151	135.04443	+	+	+
3-Hydroxyphenylacetic acid	$C_8H_8O_3$	4.93	151.0400	151.0396	0.6296	107.04962	+	+	+
Hippuric acid	$C_9H_9NO_3$	4.95	178.0510	178.0507	0.8200	134.02443	+	−	+
Ellagic acid	$C_{14}H_6O_8$	4.96	300.9989	300.9981	0.2359	229.01308, 257.00789	+	+	+
Feruloylquinic acid	$C_{17}H_{20}O_9$	4.97	367.1035	367.1013	−1.0392	191.03452, 193.05005	+	−	+
Ferulic acid-*O*-glucoside	$C_{16}H_{20}O_9$	4.98	355.1035	355.1015	−0.8886	149.02374, 178.02180, 193.04991	+	−	+
Hydroxybenzaldehyde	$C_7H_6O_2$	4.99	121.0290	121.0289	0.5341	n.d.	+	+	−
Caffeic acid-*O*-glucoside	$C_{15}H_{18}O_9$	5.02	341.0878	341.0861	−0.6585	179.03416	+	−	+
Syringic acid	$C_9H_{10}O_5$	5.03	197.0455	197.0448	0.3200	137.02895, 153.05553	+	+	+
(*Epi*)catechin-*O*-glucoside	$C_{21}H_{24}O_{11}$	5.04	451.1246	451.1227	−0.8384	245.08114, 289.06996	+	−	+
Phenylacetic acid	$C_8H_8O_2$	5.06	135.0446	135.0445	0.4841	91.04952	+	+	+
(*Epi*)gallocatechin-*O*-gallate	$C_{22}H_{18}O_{11}$	5.07	457.0776	457.0753	−1.2876	169.01328, 305.06499, 331.04422	+	−	+
Hydroxyphenylpropionic acid	$C_9H_{10}O_3$	5.08	165.0557	165.0553	0.6887	121.02880	+	+	+
ρ-Coumaric acid	$C_9H_8O_3$	5.09	163.0400	163.0396	0.6685	119.04964	+	+	+
Sinapic acid	$C_{11}H_{12}O_5$	5.10	223.0611	223.0601	0.0200	163.03972, 179.03441, 205.04986	+	−	+
Dihydroferulic acid	$C_{10}H_{12}O_4$	5.11	195.0662	195.0654	0.2046	135.04438, 151.07554	−	−	+
Ferulic acid	$C_{10}H_{10}O_4$	5.12	193.0506	193.0500	0.4548	149.05934, 179.02184	+	+	+
Secoisolariciresinol	$C_{20}H_{26}O_6$	5.13	361.1657	361.1637	−0.8245	165.05528	+	−	+
Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	5.14	515.1194	515.1168	−1.5818	191.03438, 353.08569	+	−	+
(*Epi*)catechin-*O*-gallate	$C_{22}H_{18}O_{10}$	5.15	441.0827	441.0811	−0.5427	169.01344, 289.07015, 331.04422	+	−	+
Sinapoylquinic acid	$C_{18}H_{22}O_{10}$	5.18	397.1135	397.1119	−1.0333	179.03434, 223.06015	+	−	+
Methyl-(*epi*)catechin	$C_{16}H_{16}O_6$	5.20	303.0874	303.0864	0.1146	179.03967, 289.08960	+	+	+
o-Coumaric acid	$C_9H_8O_3$	5.21	163.0400	163.0393	0.2988	119.04947	+	−	+
Coumaroylquinic acid	$C_{16}H_{18}O_8$	5.23	337.0929	337.0911	−0.6840	163.03964, 191.03441	+	−	+

(*continued on next page*)

Table 2 (*continued*)

Compound	Neutral molecular formula	Rt (min)	Ion mass $[M-H]^-$ Theoretical	Experimental	Error (amu)	MS^2 fragment ions $[M-H]^-$	*In-vitro* digestion Gastric	Intestinal	Undigested CS extract
(*Epi*)gallocatechin	$C_{15}H_{14}O_7$	5.24	305.0667	305.0650	−0.6186	179.03431, 219.02887, 221.04453	+	+	+
Coumaric acid-*O*-glucoside	$C_{15}H_{18}O_8$	5.25	325.0928	325.0918	−0.4639	119.04964, 163.03966	+	−	+
3-Phenylpropionic acid	$C_9H_{10}O_2$	5.39	149.0608	149.0598	0.0637	105.06894	+	+	+
Laricresinol	$C_{20}H_{24}O_6$	5.48	359.1495	359.1484	−0.5554	329.13818	+	+	+

+, compound identified in samples; −, compound not identified in samples. n.d., not determined. CS, chestnut shells.

of quinic acid should be investigated in further studies to support this explanation. The total concentration of hydroxycinnamic acids decreased in the same order as hydroxybenzoic acids. A higher bioaccessibility was determined for hydroxycinnamic acids after intestinal digestion (44.14%) when compared to hydroxybenzoic acids.

Only two phenylacetic acids were identified and quantified in both digests and undigested extract. 3-hydroxyphenylacetic acid was determined at higher levels than phenylacetic acid, with increasing concentrations in the following order: intestinal digest < gastric digest < undigested extract. Indeed, phenylacetic and 3-hydroxyphenylacetic acids are metabolites of caffeoylquinic, feruloylquinic, caffeic, and ferulic acids, which explains the recovery rates above 100% (Pinto, Ferreira, et al., 2023; Pinto, López-Yerena, et al., 2023).

Phenylpropanoic acids were probably the major metabolites of hydroxycinnamic acids (i.e., caffeoylquinic, feruloylquinic, caffeic, and ferulic acids) and flavanols (i.e., (*epi*)catechin, (*epi*)catechin-*O*-gallate, (*epi*)gallocatechin, and (*epi*)gallocatechin-*O*-gallate), which may justify the recovery rates of hydroxyphenylpropionic acid above 100% after gastric and intestinal digestion (Pint o , Ló pez-Yerena, et al., 2023). The highest concentrations of hydroxyphenylpropionic acid in the intestinal digest agree with the lowest concentrations of hydroxycinnamic acids and flavanols, suggesting that this metabolite originated during the intestinal phase by the action of gut enzymes and neutral pH. In addition, dihydroxyphenylpropionic acid was not quantified in the undigested extract, reinforcing its role as a phenolic metabolite produced during *in-vitro* digestion.

Flavanols were the only subclass of flavonoids identified at significantly ($p < 0.05$) lower concentrations after digestion. Only trace levels of (*epi*)catechin, (*epi*)gallocatechin, and methyl-(*epi*)catechin were determined.

Ellagic acid was the only hydrolyzable tannin identified, revealing a significantly ($p < 0.05$) higher concentration in the undigested extract (2.52 μg/mg DW), followed by gastric (0.26 μg/mg DW) and intestinal (0.05 μg/mg DW) digests, with low recovery rates (10.34% and 2.16%, respectively). These results suggest the extensive metabolization of ellagic acid by the intestinal microbiota probably into urolithins due to its instability under gastric and intestinal conditions (Pinto, Almeida, et al., 2023).

Concerning the lignans, laricresinol was identified in the digested and undigested extract, with decreasing concentrations from the undigested extract (0.05 μg/mg DW) to its intestinal digest (0.01 μg/mg DW). Secoisolariciresinol was identified only in the undigested extract and its gastric digest. Lignans are also metabolized by the intestinal microbiota, originating enterolignans (i.e., enterodiol and enterolactone) with proven pro-healthy benefits, including antioxidant, anticancer, and modulating effects on hormone metabolism (Pinto, López-Yerena, et al., 2023).

Moreover, other phenolic compounds were quantified, including catechol, pyrogallol, and hydroxybenzaldehyde, revealing higher concentrations in the undigested extract and lower after intestinal digestion. Noteworthy, hydroxybenzaldehyde was quantified only in the digests, proposing that this compound is a metabolite derived from the reduction of hydroxybenzoic acids during digestion (Bento-Silva et al., 2020).

Hippuric acid and hydroxyhippuric acid were also identified as phenolic metabolites in the digested fractions of CS extract. Hippuric acid is an acyl glycine formed by the conjugation of benzoic acid and glycine (Pinto, López-Yerena, et al., 2023). The benzoic acid may be derived from quinic acid released by hydrolysis of caffeoylquinic and galloylquinic acids (Pinto, Ferreira, et al., 2023). However, it was not possible to quantify these phenolic metabolites.

Besides the identical *in-vitro* bioactivity proven by previous assays, the present results reinforced the distinct phenolic profile of CS extract and its gastric and intestinal digests, allowing to understand the molecular modifications and degradation of phenolic compounds that occur during metabolism by digestive enzymes under acidic and alkaline conditions. Furthermore, certain phenolics may bind to proteins under acidic and alkaline conditions via covalent, hydrogen, and hydrophobic bonds, reducing their concentrations (Hu et al., 2023). Overall, the decrease of total phenolic concentrations after intestinal digestion can be associated with the sensitivity of these molecules to higher pH values, becoming less stable and leading to their hydrolysis into smaller molecules during digestion.

3.8. Multivariate data analysis

Multivariate data analysis was performed by PCA and heatmap correlation to outline differences between undigested CS extract and respective gastric and intestinal digests (Fig. 3).

Considering Fig. 3A, the scores plot indicates three individual clusters with 92.19% of cumulative variance. The principal component (PC) 1 justifies the major fraction of the variance of the results (70.88%), while the remaining 21.31% of the variance is explained by PC2. The differences between undigested extract and both gastric and intestinal digests are mostly elucidated by PC1, while PC2 explains the variance between gastric and intestinal digests. In summary, the PCA model points out distinct responses for the undigested CS extract and its gastric and intestinal digests, highlighting clear differences in the metabolic profile, bioaccessibility, and bioactivity of phenolic compounds extracted from CS before and after *in-vitro* digestion.

The heatmap correlations depicted in Fig. 3B–3D highlight positive correlations between most of the variables studied for the undigested CS extract and its gastric and intestinal digests. Even though different correlation patterns were observed between samples, these results emphasize the exceptional role of phenolic compounds and flavonoids (namely hydroxycinnamic acids, hydroxybenzoic acids, phenylpropanoic acids, phenylacetic acids, flavanols, and hydrolyzable tannins) in the antioxidant/antiradical effects, radicals scavenging efficiency, activation of antioxidant enzymes activities, protection against LPO, and hypoglycemic and neuroprotective properties of CS extract before and after *in-vitro* digestion, exhibiting mild to strong correlations. In general, significant correlations ($p < 0.05$) were mainly obtained between ROS and RNS scavenging potential, SOD, CAT and GSH-Px activities, LPO, and AChE and α-amylase inhibitory activity with the polyphenolic classes identified in the undigested and digested CS extract, highlighting a remarkable contribution of these phenolic compounds to the bioactivity of CS extract and its digests. In summary, the

Table 3
Quantification of phenolic compounds and their metabolites in undigested chestnut shells (CS) extract and respective gastric and intestinal digests by LC-ESI-LTQ-Orbitrap-MS.

Phenolic compounds	*In-vitro* digestion				Undigested CS extract (μg/mg DW)
	Gastric		Intestinal		
	Amount (μg/mg DW)	Recovery (%)	Amount (μg/mg DW)	Recovery (%)	
Phenolic acids – Hydroxybenzoic acids and derivatives					
3-Hydroxybenzoic acid	2.47 ± 0.05^{a}	115.89 ± 2.50^{1}	2.00 ± 0.10^{b}	94.19 ± 4.49^{2}	2.13 ± 0.10^{b}
4-Hydroxybenzoic acid	0.44 ± 0.03^{b}	38.89 ± 2.52^{1}	0.33 ± 0.01^{c}	29.12 ± 1.23^{2}	1.13 ± 0.01^{a}
2,5-Dihydroxybenzoic acid	0.01 ± 0.00^{a}	124.82 ± 7.48	n.i.	–	0.01 ± 0.00^{a}
3,5-Dihydroxybenzoic acid	0.46 ± 0.01^{a}	160.63 ± 3.24^{1}	0.05 ± 0.00^{c}	16.07 ± 0.36^{2}	0.29 ± 0.00^{b}
Gallic acid	5.00 ± 0.21^{b}	54.54 ± 2.26^{1}	0.37 ± 0.02^{c}	4.08 ± 0.17^{2}	9.17 ± 0.34^{a}
Protocatechuic acid	n.i.	–	n.i.	–	1.66 ± 0.05
Syringic acid	0.81 ± 0.01^{a}	165.32 ± 1.66^{1}	0.08 ± 0.00^{c}	16.62 ± 0.32^{2}	0.49 ± 0.04^{b}
Galloylquinic acid	n.i.	–	n.i.	–	0.01 ± 0.00
Hydroxybenzoic acid-*O*-glucoside	0.01 ± 0.00^{a}	66.80 ± 2.05	n.i.	–	0.01 ± 0.00^{a}
Methyl gallate	0.06 ± 0.00^{b}	41.30 ± 1.05^{1}	0.01 ± 0.00^{c}	3.63 ± 0.24^{2}	0.15 ± 0.01^{a}
Monogalloyl glucose	n.i.	–	n.i.	–	0.05 ± 0.00
Protocatechuic acid-*O*-glucoside	n.i.	–	n.i.	–	0.01 ± 0.00
Syringic acid-*O*-glucoside	0.01 ± 0.00^{b}	50.16 ± 8.46	n.i.	–	0.02 ± 0.00^{a}
∑ Hydroxybenzoic acids	9.28 ± 0.31^{b}	61.25 ± 2.05^{1}	2.85 ± 0.13^{c}	18.83 ± 0.85^{2}	15.15 ± 0.23^{a}
Phenolic acids – Hydroxycinnamic acids and derivatives					
Caffeic acid	0.07 ± 0.01^{a}	96.11 ± 8.81^{1}	0.03 ± 0.00^{b}	41.49 ± 1.03^{2}	0.07 ± 0.00^{a}
Chlorogenic acid	0.01 ± 0.00^{b}	60.58 ± 2.36^{2}	0.02 ± 0.00^{a}	127.99 ± 3.50^{1}	0.01 ± 0.00^{b}
Sinapic acid	0.01 ± 0.00^{a}	116.42 ± 9.25	n.i.	–	0.01 ± 0.00^{a}
p-Coumaric acid	0.07 ± 0.00^{b}	110.74 ± 3.16^{2}	0.08 ± 0.00^{a}	127.49 ± 1.58^{1}	0.06 ± 0.00^{c}
Ferulic acid	0.03 ± 0.01^{a}	86.24 ± 15.92^{1}	0.02 ± 0.00^{b}	53.15 ± 7.37^{2}	0.03 ± 0.00^{a}
Dihydroferulic acid	n.i.	–	n.i.	–	0.06 ± 0.00
Sinapic acid-*O*-glucoside	0.01 ± 0.00^{b}	16.29 ± 1.15	n.i.	–	0.06 ± 0.00^{a}
Caffeic acid-*O*-glucoside	n.q.	–	n.i.	–	0.01 ± 0.00
Ferulic acid-*O*-glucoside	0.01 ± 0.00^{b}	45.25 ± 0.86	n.i.	–	0.02 ± 0.00^{a}
Coumaric acid-*O*-glucoside	0.01 ± 0.00^{b}	23.94 ± 1.81	n.i.	–	0.03 ± 0.00^{a}
Coumaroylquinic acid	0.01 ± 0.00^{a}	56.32 ± 0.09	n.i.	–	0.01 ± 0.00^{a}
Feruloylquinic acid	0.01 ± 0.00^{b}	50.02 ± 3.56	n.i.	–	0.02 ± 0.00^{a}
Sinapoylquinic acid	n.q.	–	n.i.	–	0.02 ± 0.00
∑ Hydroxycinnamic acids	0.25 ± 0.02^{b}	56.73 ± 4.04^{1}	0.19 ± 0.00^{c}	44.14 ± 0.33^{2}	0.43 ± 0.00^{a}
Phenolic acids – Phenylacetic acids					
3-Hydroxyphenylacetic acid	5.17 ± 0.18^{b}	82.44 ± 2.88^{1}	2.33 ± 0.06^{c}	37.26 ± 1.01^{2}	6.27 ± 0.04^{a}
Phenylacetic acid	0.15 ± 0.01^{a}	145.19 ± 8.67^{1}	0.15 ± 0.01^{a}	140.65 ± 11.97^{1}	0.10 ± 0.00^{b}
∑ Phenylacetic acids	5.32 ± 0.19^{b}	83.46 ± 2.97^{1}	2.48 ± 0.08^{c}	38.93 ± 1.19^{2}	6.37 ± 0.04^{a}
Phenolic acids – Phenylpropanoic acids					
Hydroxyphenylpropionic acid	19.97 ± 0.80^{b}	104.73 ± 4.17^{2}	29.36 ± 0.46^{a}	153.95 ± 2.39^{1}	19.07 ± 1.31^{b}
Dihydroxyphenylpropionic acid	0.02 ± 0.00^{a}	–	0.01 ± 0.00^{b}	–	n.i.
Phenylpropionic acid	1.04 ± 0.09^{b}	70.82 ± 5.81^{1}	0.37 ± 0.01^{c}	25.10 ± 0.48^{2}	1.47 ± 0.07^{a}
∑ Phenylpropanoic acids	21.03 ± 0.88^{b}	102.39 ± 4.29^{2}	29.73 ± 0.45^{a}	144.77 ± 2.19^{1}	20.54 ± 1.38^{b}
∑ Phenolic acids	35.87 ± 1.40^{b}	84.42 ± 3.29^{1}	35.26 ± 0.25^{b}	82.98 ± 0.58^{1}	42.49 ± 1.19^{a}
Flavonoids – Flavanols					
(*Epi*)catechin	0.01 ± 0.00^{b}	36.32 ± 0.43	n.q.	–	0.02 ± 0.00^{a}
(*Epi*)gallocatechin	0.01 ± 0.00^{b}	71.16 ± 1.12^{1}	0.01 ± 0.00^{c}	57.15 ± 1.24^{2}	0.01 ± 0.00^{a}
Methyl-(*epi*)catechin	0.01 ± 0.00^{b}	46.73 ± 2.38	n.q.	–	0.01 ± 0.00^{a}
∑ Flavonoids	0.02 ± 0.00^{b}	46.50 ± 2.07^{1}	0.01 ± 0.00^{c}	22.68 ± 0.62^{2}	0.04 ± 0.00^{a}
Hydrolysable tannins					
Ellagic acid	0.26 ± 0.01^{b}	10.34 ± 0.31^{1}	0.05 ± 0.00^{c}	2.16 ± 0.06^{2}	2.52 ± 0.23^{a}
Lignans					
Lariciresinol	0.05 ± 0.00^{a}	105.62 ± 8.58^{1}	0.01 ± 0.00^{b}	11.65 ± 1.43^{2}	0.05 ± 0.00^{a}
Other phenolics					
Catechol	0.08 ± 0.00^{a}	129.93 ± 4.92^{1}	0.01 ± 0.00^{c}	16.98 ± 0.07^{2}	0.06 ± 0.00^{b}
Pyrogallol	0.33 ± 0.01^{b}	59.38 ± 2.04^{1}	0.06 ± 0.00^{c}	11.56 ± 0.22^{2}	0.56 ± 0.01^{a}
Hydroxybenzaldehyde	0.12 ± 0.00^{b}	–	0.14 ± 0.00^{a}	–	n.i.
∑ Other phenolics	0.53 ± 0.02^{b}	86.16 ± 2.83^{1}	0.21 ± 0.00^{c}	34.13 ± 0.32^{2}	0.61 ± 0.01^{a}
∑ Phenolic compounds	36.75 ± 1.43^{b}	80.29 ± 3.12^{1}	35.54 ± 0.25^{b}	77.64 ± 0.54^{1}	45.78 ± 0.94^{a}

n.i., non-identified. n.q., non-quantified. CS, chestnut shells. DW, dry weight. Results are expressed as mean ± standard deviation ($n = 3$). Different letters (a, b, and c) in the same line indicate significant differences between groups ($p < 0.05$) regarding phenolics concentrations. Different numbers at superscript (1, and 2) in the same line indicate significant differences between groups ($p < 0.05$) regarding recovery rates.

metabolic profile of phenolic compounds greatly contributes to the antioxidant/antiradical properties, ROS/RNS scavenging capacity, activation of CAT and GSH-Px activities, and anti-cholinergic activity, offering protective effects against oxidative stress injuries.

The multivariate statistical analysis indicates a pronounced heterogeneity between the undigested CS extract and its gastric and intestinal digests considering the *in-vitro* bioactivity and bioaccessibility of phenolic compounds. Furthermore, the phenolic concentrations and their bioactivity can fluctuate in response to different metabolic pathways that affect their bioaccessibility.

4. Conclusion

This study attempted, for the first time, to evaluate the impact of *in-vitro* gastrointestinal digestion in the bioaccessibility of phenolic compounds extracted from CS by SFE and their biological activities,

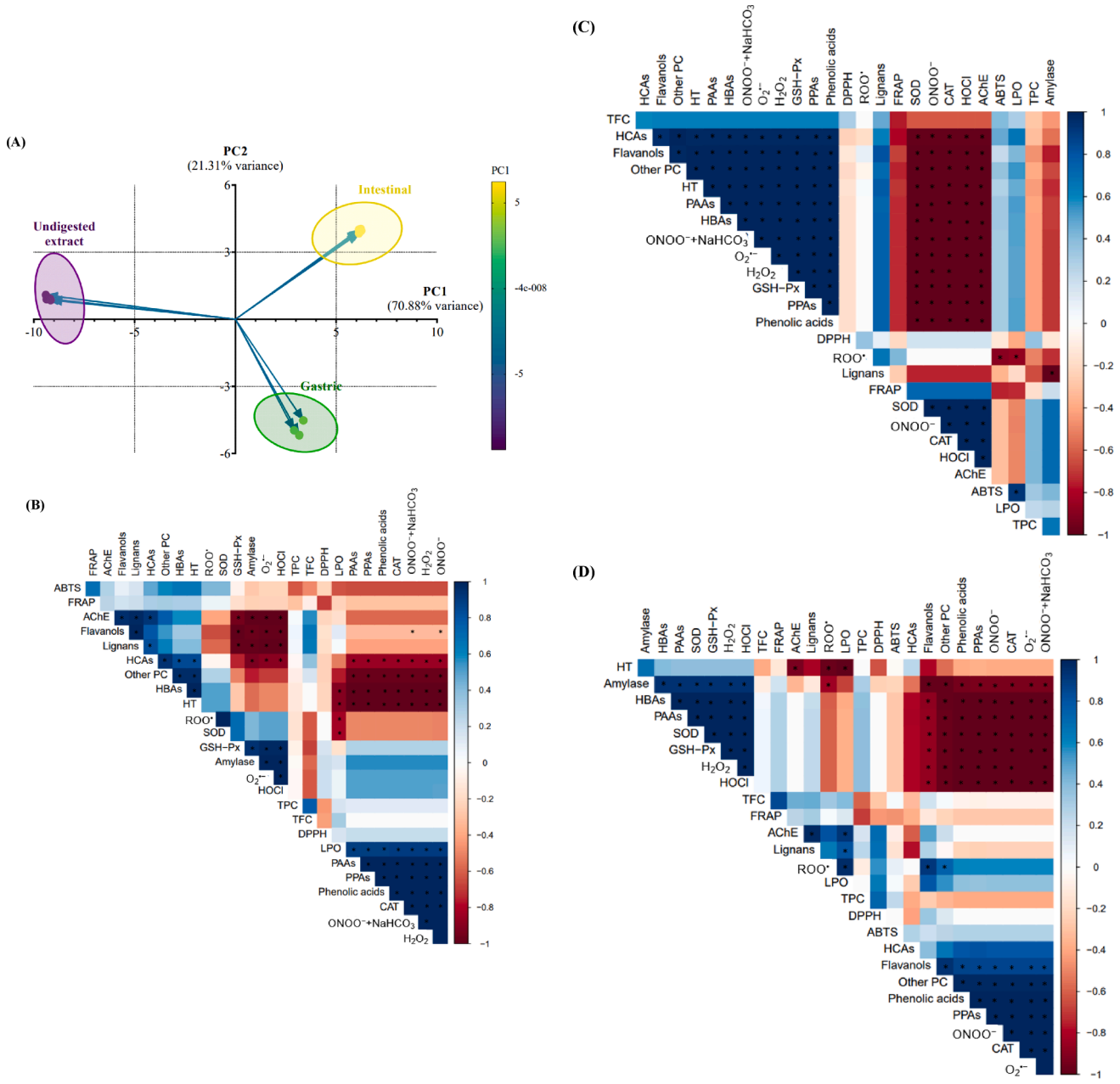

Fig. 3. Multivariate data analysis on metabolic profiling targeted on phenolics and bioactivity of chestnut shells (CS) extract before and after *in-vitro* digestion: scores plot (A), and heatmap correlation diagrams for undigested extract (B), gastric digest (C), and intestinal digest (D). * denotes significant differences ($p < 0.05$) between two variables. AChE, acetylcholinesterase. CAT, catalase. FRAP, ferric reducing antioxidant power. GSH-Px, glutathione peroxidase. HBAs, hydroxybenzoic acids. HCAs, hydroxycinnamic acids. HT, hydrolysable tannins. LPO, lipid peroxidation. PAAs, phenylacetic acids. PC, phenolic compounds. PPAs, phenylpropanoic acids. SOD, superoxide dismutase. TFC, total flavonoid content. TPC, total phenolic content.

exploring the metabolic profile by LC-ESI-LTQ-Orbitrap-MS and considering a final application as a nutraceutical ingredient. The main findings of this study were:

- The phenolic concentrations retained after gastric and intestinal digestion decreased significantly when compared to the undigested extract, reaching a maximum bioaccessibility of 30%.
- The metabolic profile revealed the biotransformation of complex phenolic acids and flavonoids into smaller metabolites, mainly phenylpropanoic acids.
- Gallic acid, ellagic acid, 3-hydroxyphenylacetic acid, and hydroxyphenylpropionic acid were the main phenolic compounds identified in the digested and undigested fractions.
- Despite the higher bioactivity of the undigested extract, its gastric and intestinal digests still exhibited promising antioxidant/antiradical, hypoglycemic, and neuroprotective properties, as well as upmodulating effects of antioxidant enzymes activities, and protection against LPO.
- PCA and heatmap pointed out the exceptional contribution of the phenolic composition to the bioactivity of CS extract upon simulated digestion, along with distinct responses for the undigested extract and its gastric and intestinal digests.

These findings prove the antioxidant efficacy of the phenolics-rich CS extract prepared by SFE even after gastrointestinal digestion, highlighting the pro-healthy benefits of its incorporation into functional foods and nutraceuticals. Further research should focus on

encapsulation techniques as effective delivery agents of phenolic compounds, aiming to improve their bioaccessibility and, consequently, bioactivity.

CRediT authorship contribution statement

Diana Pinto: Methodology, Software, Formal analysis, Investigation, Writing – original draft. **Anallely López-Yerena:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Rosa Lamuela-Raventós:** Methodology, Funding acquisition, Resources. **Anna Vallverdú-Queralt:** Methodology, Supervision, Writing – review & editing. **Cristina Delerue-Matos:** Methodology, Supervision, Resources. **Francisca Rodrigues:** Methodology, Conceptualization, Validation, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137581.

References

Alvarez-Rodríguez, M. L., Belloch, C., Villa, M., Uruburu, F., Larriba, G., & Coque, J. J. (2003). Degradation of vanillic acid and production of guaiacol by microorganisms isolated from cork samples. *FEMS Microbiology Letters, 220*, 49–55. https://doi.org/10.1016/s0378-1097(03)00053-3

Andishmand, H., Azadmard-damirchi, S., Hamishekar, H., Torbati, M., Kharazmi, M. S., Savage, G. P., Tan, C., & Jafari, S. M. (2023). Nano-delivery systems for encapsulation of phenolic compounds from pomegranate peel. *Advances in Colloid and Interface Science, 311*, Article 102833. https://doi.org/10.1016/j.cis.2022.102833

Andrade, J. K. S., Barros, R. G. C., Pereira, U. C., Gualberto, N. C., de Oliveira, C. S., Shanmugam, S., & Narain, N. (2022). α-Amylase inhibition, cytotoxicity and influence of the *in vitro* gastrointestinal digestion on the bioaccessibility of phenolic compounds in the peel and seed of *Theobroma grandiflorum*. *Food Chemistry, 373*, Article 131494. https://doi.org/10.1016/j.foodchem.2021.131494

Bento-Silva, A., Koistinen, V. M., Mena, P., Bronze, M. R., Hanhineva, K., Sahlstrøm, S., Kitrytė, V., Moco, S., & Aura, A. M. (2020). Factors affecting intake, metabolism and health benefits of phenolic acids: Do we understand individual variability? *European Journal of Nutrition, 59*, 1275–1293. https://doi.org/10.1007/s00394-019-01987-6

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant power": the FRAP assay. *Analytical Biochemistry, 239*, 70–76. https://doi.org/10.1006/abio.1996.0292

de Vasconcelos, M. d. C. B. M., Bennett, R. N., Quideau, S., Jacquet, R., Rosa, E. A. S., & Ferreira-Cardoso, J. V. (2010). Evaluating the potential of chestnut (*Castanea sativa* Mill.) fruit pericarp and integument as a source of tocopherols, pigments and polyphenols. *Industrial Crops and Products*, *31*, 301-311. Doi: 10.1016/j.indcrop.2009.11.008.

Ferreira, A. S., Silva, A. M., Pinto, D., Moreira, M. M., Ferraz, R., Švarc-Gajić, J., Costa, P. C., Delerue-Matos, C., & Rodrigues, F. (2022). New perspectives on the sustainable employment of chestnut shells as active ingredient against oral mucositis: A first screening. *International Journal of Molecular Sciences, 23*, 14956. https://doi.org/10.3390/ijms232314956

Gawlik-Dziki, U. (2014). Modification of enzymatic and non-enzymatic *in vitro* oxidative defence system by bioaccessible phytonutrients of selected spices. *LWT - Food Science and Technology, 57*, 434–441. https://doi.org/10.1016/j.lwt.2013.12.039

Gomes, A., Fernandes, E., Silva, A. M. S., Santos, C. M. M., Pinto, D. C. G. A., Cavaleiro, J. A. S., & Lima, J. L. F. C. (2007). 2-Styrylchromones: Novel strong scavengers of reactive oxygen and nitrogen species. *Bioorganic & Medicinal Chemistry, 15*, 6027–6036. https://doi.org/10.1016/j.bmc.2007.06.046

Hatano, T., Kagawa, H., Yasuhara, T., & Okuda, T. (1988). Two new flavonoids and other constituents in licorice root: Their relative astringency and radical scavenging effects. *Chemical and Pharmaceutical Bulletin, 36*, 2090–2097. https://doi.org/10.1248/cpb.36.2090

Hu, Y., Lin, Q., Zhao, H., Li, X., Sang, S., McClements, D. J., Long, J., Jin, Z., Wang, J., & Qiu, C. (2023). Bioaccessibility and bioavailability of phytochemicals: Influencing factors, improvements, and evaluations. *Food Hydrocolloids, 135*, Article 108165. https://doi.org/10.1016/j.foodhyd.2022.108165

Kashyap, P., Riar, C. S., & Jindal, N. (2022). Effect of extraction methods and simulated *in vitro* gastrointestinal digestion on phenolic compound profile, bio-accessibility, and antioxidant activity of Meghalayan cherry (*Prunus nepalensis*) pomace extracts. *LWT - Food Science and Technology, 153*, Article 112570. https://doi.org/10.1016/j.lwt.2021.112570

Ketsawatsakul, U., Whiteman, M., & Halliwell, B. (2000). A reevaluation of the peroxynitrite scavenging activity of some dietary phenolics. *Biochemical & Biophysical Research Communications, 279*, 692–699. https://doi.org/10.1006/bbrc.2000.4014

Lameirão, F., Pinto, D., F. Vieira, E., F. Peixoto, A., Freire, C., Sut, S., Dall'Acqua, S., Costa, P., Delerue-Matos, C., & Rodrigues, F. (2020). Green-sustainable recovery of phenolic and antioxidant compounds from industrial chestnut shells using ultrasound-assisted extraction: Optimization and evaluation of biological activities *in vitro*. *Antioxidants*, *9*, 267. doi: 10.3390/antiox9030267.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., … Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food - An international consensus. *Food & Function, 5*, 1113–1124. https://doi.org/10.1039/c3fo60702j

Muñoz-Fariña, O., López-Casanova, V., García-Figueroa, O., Roman-Benn, A., Ah-Hen, K., Bastias-Montes, J. M., … Ravanal-Espinosa, M. C. (2023). Bioaccessibility of phenolic compounds in fresh and dehydrated blueberries (*Vaccinium corymbosum* L.). *Food Chemistry Advances, 2*, Article 100171. https://doi.org/10.1016/j.focha.2022.100171

Murugan, R., Chandran, R., & Parimelazhagan, T. (2016). Effect of *in vitro* simulated gastrointestinal digestion of *Phoenix loureirii* on polyphenolics, antioxidant and acetylcholinesterase inhibitory activities. *LWT - Food Science and Technology, 74*, 363–370. https://doi.org/10.1016/j.lwt.2016.07.075

Peláez-Acero, A., Garrido-Islas, D. B., Campos-Montiel, R. G., González-Montiel, L., Medina-Pérez, G., Luna-Rodríguez, L., … d. J.. (2022). The application of ultrasound in honey: Antioxidant activity, inhibitory effect on α-amylase and α-glucosidase, and *in vitro* digestibility assessment. *Molecules, 27*, 5825. https://doi.org/10.3390/molecules27185825

Pinto, D., Almeida, A., López-Yerena, A., Pinto, S., Sarmento, B., Lamuela-Raventós, R., … Rodrigues, F. (2023). Appraisal of a new potential antioxidants-rich nutraceutical ingredient from chestnut shells through *in-vivo* assays – A targeted metabolomic approach in phenolic compounds. *Food Chemistry, 404*, Article 134546. https://doi.org/10.1016/j.foodchem.2022.134546

Pinto, D., Cádiz-Gurrea, M.d.l. L., Garcia, J., Saavedra, M. J., Freitas, V., Costa, P., … Rodrigues, F. (2021). From soil to cosmetic industry: Validation of a new cosmetic ingredient extracted from chestnut shells. *Sustainable Materials and Technologies, 29*, Article e00309. https://doi.org/10.1016/j.susmat.2021.e00309

Pinto, D., Cádiz-Gurrea, M.d.l. L., Sut, S., Ferreira, A. S., Leyva-Jimenez, F. J., Dall'Acqua, S., … Rodrigues, F. (2020). Valorisation of underexploited *Castanea sativa* shells bioactive compounds recovered by supercritical fluid extraction with CO_2: A response surface methodology approach. *Journal of CO2 Utilization, 40*, 101194. https://doi.org/10.1016/j.jcou.2020.101194

Pinto, D., Cádiz-Gurrea, M.d.l. L., Vallverdú-Queralt, A., Delerue-Matos, C., & Rodrigues, F. (2021). *Castanea sativa* shells: A review on phytochemical composition, bioactivity and waste management approaches for industrial valorization. *Food Research International, 144*, Article 110364. https://doi.org/10.1016/j.foodres.2021.110364

Pinto, D., Ferreira, A. S., Lozano-Castellón, J., Laveriano-Santos, E. P., Lamuela-Raventós, R. M., Vallverdú-Queralt, A., … Rodrigues, F. (2023). Exploring the impact of *in vitro* gastrointestinal digestion in the bioaccessibility of phenolic-rich chestnut shells: A preliminary study. *Separations, 10*, 471. https://doi.org/10.3390/separations10090471

Pinto, D., López-Yerena, A., Almeida, A., Sarmento, B., Lamuela-Raventós, R., Vallverdú-Queralt, A., … Rodrigues, F. (2023). Metabolomic insights into phenolics-rich chestnut shells extract as a nutraceutical ingredient – A comprehensive evaluation of its impacts on oxidative stress biomarkers by an *in-vivo* study. *Food Research International, 170*, Article 112963. https://doi.org/10.1016/j.foodres.2023.112963

Pinto, D., Moreira, M. M., Švarc-Gajić, J., Vallverdú-Queralt, A., Brezo-Borjan, T., Delerue-Matos, C., & Rodrigues, F. (2023). *In-vitro* gastrointestinal digestion of functional cookies enriched with chestnut shells extract: Effects on phenolic composition, bioaccessibility, bioactivity, and α-amylase inhibition. *Food Bioscience, 53*, Article 102766. https://doi.org/10.1016/j.fbio.2023.102766

Pinto, D., Moreira, M. M., Vieira, E. F., Švarc-Gajić, J., Vallverdú-Queralt, A., Brezo-Borjan, T., Delerue-Matos, C., & Rodrigues, F. (2023). Development and characterization of functional cookies enriched with chestnut shells extract as source of bioactive phenolic compounds. *Foods, 12*, 640. https://doi.org/10.3390/foods12030640

Pinto, D., Reis, J., Silva, A. M., Salazar, M., Dall'Acqua, S., Delerue-Matos, C., & Rodrigues, F. (2021). Valorisation of *Salicornia ramosissima* biowaste by a green approach – An optimizing study using response surface methodology. *Sustainable Chemistry and Pharmacy, 24*, Article 100548. https://doi.org/10.1016/j.scp.2021.100548

Pinto, D., Silva, A. M., Dall'Acqua, S., Sut, S., Vallverdú-Queralt, A., Delerue-Matos, C., & Rodrigues, F. (2023). Simulated gastrointestinal digestion of chestnut (*Castanea sativa* Mill.) shell extract prepared by subcritical water extraction: Bioaccessibility, bioactivity, and intestinal permeability by *in vitro* assays. *Antioxidants, 12*, 1414. https://doi.org/10.3390/antiox12071414

Pinto, D., Silva, A. M., Freitas, V., Vallverdú-Queralt, A., Delerue-Matos, C., & Rodrigues, F. (2021). Microwave-assisted extraction as a green technology approach to recover polyphenols from *Castanea sativa* shells. *ACS Food Science & Technology, 1*, 229–241. https://doi.org/10.1021/acsfoodscitech.0c00055

Pinto, D., Vieira, E. F., Peixoto, A. F., Freire, C., Freitas, V., Costa, P., Delerue-Matos, C., & Rodrigues, F. (2021). Optimizing the extraction of phenolic antioxidants from chestnut shells by subcritical water extraction using response surface methodology. *Food Chemistry, 334*, Article 127521. https://doi.org/10.1016/j.foodchem.2020.127521

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*, 1231–1237. https://doi.org/10.1016/s0891-5849(98)00315-3

Ruíz-García, Y., Beres, C., Chávez, D. W. H., Pereira, D. C.d. S., Santiago, M. C. P. A., Godoy, R.d. O., … Cabral, L. M. C. (2022). *In vitro* digestion and colonic fermentation of an Alicante Bouschet (*Vitis vinifera* L.) skin extract. *LWT - Food Science and Technology, 157*, 113083. https://doi.org/10.1016/j.lwt.2022.113083

Sangiovanni, E., Piazza, S., Vrhovsek, U., Fumagalli, M., Khalilpour, S., Masuero, D., Di Lorenzo, C., Colombo, L., Mattivi, F., De Fabiani, E., & Dell'Agli, M. (2018). A bio-guided approach for the development of a chestnut-based proanthocyanidin-enriched nutraceutical with potential anti-gastritis properties. *Pharmacological Research, 134*, 145–155. https://doi.org/10.1016/j.phrs.2018.06.016

Chitosan suspension as extractor and encapsulating agent of phenolics from acerola by-productSilva, N. C.d., Assis, O. B. G., Sartori, A. G.d. O., Alencar, S. M.d., & Martelli-Tosi, M. (Eds.). *Food Research International, 161*, (2022), 111855. https://doi.org/10.1016/j.foodres.2022.111855

Singleton, V. L., Rossi, J. A., & Jr.. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144. https://doi.org/10.5344/ajev.1965.16.3.144

Sova, M., & Saso, L. (2020). Natural sources, pharmacokinetics, biological activities and health benefits of hydroxycinnamic acids and their metabolites. *Nutrients, 12*, 2190. https://doi.org/10.3390/nu12082190

Tsujita, T., Takaku, T., & Suzuki, T. (2008). Chestnut astringent skin extract, an α-amylase inhibitor, retards carbohydrate absorption in rats and humans. *Journal of Nutritional Science and Vitaminology, 54*, 82–88. https://doi.org/10.3177/jnsv.54.82

Tu, F., Xie, C., Li, H., Lei, S., Li, J., Huang, X., & Yang, F. (2021). Effect of *in vitro* digestion on chestnut outer-skin and inner-skin bioaccessibility: The relationship between biotransformation and antioxidant activity of polyphenols by metabolomics. *Food Chemistry, 363*, Article 130277. https://doi.org/10.1016/j.foodchem.2021.130277

Wu, H., Liu, Z., Lu, P., Barrow, C., Dunshea, F. R., & Suleria, H. A. R. (2022). Bioaccessibility and bioactivities of phenolic compounds from roasted coffee beans during *in vitro* digestion and colonic fermentation. *Food Chemistry, 386*, Article 132794. https://doi.org/10.1016/j.foodchem.2022.132794

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry, 64*, 555–559. https://doi.org/10.1016/S0308-8146(98)00102-2