Bicarbonate-assisted extraction of pomegranate peel (*Punica granatum* L.) to obtain extracts with new low molecular weight phenols and enriched in total tannins and ellagic acid

Silvia D'Agostino[1†], Tommasso Ugolini[1†], Deborah Freschini[2], Mohamad Khatib[2], Lorenzo Cecchi[1], Bruno Zanoni[1], Beatrice Zonfrillo[2], Marzia Innocenti[2], Maria Bellumori[2], Nadia Mulinacci[2*]

[1]Department of Agriculture, Food, Environment and Forestry Sciences and Technologies (DAGRI), University of Florence, Florence, Italy; [2]Department of Neurofarba, Nutraceutical Section, University of Florence, Sesto Fiorentino, Florence, Italy

[†]These authors contributed equally to this research

***Corresponding Author:** Nadia Muliacci, Department of Neurofarba, Nutraceutical Section, University of Florence, Via Ugo Schiff 6, 50019 Sesto Fiorentino, Florence, Italy. Email: nadia.mulinacci@unifi.it

Academic Editor: Prof. Simone Vincenzi, University of Padova, Italy

Received: 29 January 2025; Accepted: 29 June 2025; Published: 1 October 2025

PAPER

Abstract

Pomegranate peel is a by-product rich in ellagitannins, characterized by high molecular weight and low bioavailability. Working on several peel samples, we aimed to propose a single-step bicarbonate-assisted extraction to produce dry extracts with new phenol profiles rich in hydrolyzed tannins with potentially higher bioavailability. Compared to decoction, 0.6% sodium bicarbonate ($NaHCO_3$) for 1 h extraction allowed 3.5–6.5-fold and 5.6–11.3-fold increase in ellagic acid and gallic acid, respectively, with total phenols ranging from 28.3% to 35.1% w/w. Simple pomegranate peel extraction with bicarbonate can be applied to prepare botanical extracts suitable for the food supplement market, thus increasing the value of this by-product.

Keywords: ellagic acid; food by-products; gallic acid; polyphenols; punicalagin; HPLC-DAD-MS

Introduction

Pomegranate fruit (*Punica granatum* L.), commonly consumed as juice, is rich in phenolic derivatives, such as anthocyanins (Balli *et al.*, 2020), well-known for their anti-inflammatory, antioxidant, anti-hypertensive, and cardioprotective properties (Basu and Penugonda, 2009). For each ton of juice, approximately 4.5 tons of pomegranate peel are produced (Mourtzinos and Goula, 2019). Owing to high tannin concentration, dried peels (DP) represent a valuable by-product to produce health supplements or functional ingredients (Fahmy and Farag, 2022; Li *et al.*, 2006). Several *in vitro* and *in vivo* studies have demonstrated that pomegranate peel extracts exert bioactivities, such as antioxidant (Althunibat *et al.*, 2010; Haghighian *et al.*, 2021), anti-inflammatory (Mastrogiovanni *et al.*, 2019), antimicrobial (Dey *et al.*, 2015), meliorative of lipid metabolism (Liu *et al.*, 2015; Lv *et al.*, 2016), neuroprotective (Amri *et al.*, 2017), hepatoprotective (Akuru *et al.*, 2022; Zhai *et al.*, 2018), chemopreventive (Li *et al.*, 2016), and antidiabetic (Mo *et al.*, 2019), all associated with bioactive constituents, such as polysaccharides, anthocyanins, and hydrolysable tannins (Xiang *et al.*, 2022).

The most abundant phenol in pomegranate peel is punicalagins (Fischer *et al.*, 2011), hydrolysable tannins characterized by ellagic acid (EA) moieties, high molecular

weight (MW), and low bioavailability (Espín *et al.*, 2013). In fact, phenols uptake from the digestive tract usually occurs through free diffusion, resulting in an easier passage for low-molecular-weight phenols (Shahidi and Peng, 2018). EA is also a key driver of urolithins production (Long *et al.*, 2019), typical metabolites of punicalagin, with beneficial effects on target tissues (Venusova *et al.*, 2021). Previous research found increased antioxidant activities of hydrolyzed fermented pomegranate peels compared to non-hydrolyzed ones (Verotta *et al.*, 2018), suggesting that chemical hydrolysis could increase health-related effects of peels. Low amounts of free EA are present in pomegranate peels and simple strategies are needed to release it from high molecular weight tannins, like punicalagin and punicalin. Extracts enriched in EA could be used as food supplements or functional ingredients.

To date, few data are available in the literature on the type and amount of hydrolyzed tannins recovered from pomegranate peel after appropriate extraction procedures. García-Villalba *et al.* (2015) proposed an optimized extraction with hydrolysis procedure in strong acid environment to reach effectively a complete hydrolysis of precursors (i.e., punicalagin). Alkaline hydrolysis of pomegranate peel was proposed only by a study using a strong base agent at various concentrations (Liu *et al.*, 2013), highlighting that high concentration of NaOH caused complete loss of EA and gallic acid (GA). However, in Liu *et al.* (2013), authors did not analyze water extracts but only the diethyl ether/ethyl acetate extract, thus not considering water-soluble tannins, such as punicalin and punicalagin, and not discussing degradations that occurred on these important pomegranate peel phenols; furthermore, accurate data on the amount of total phenols and on newly produced phenols by hydrolysis from alkaline extraction were completely missing. So far, no data are present in the literature on a single-step extraction of pomegranate peel, assisted with a food-grade alkaline ingredient, such as bicarbonate.

Phenolic compounds are also present in plant matrices not in free form but linked (Zhang *et al.* 2020) with polymeric components of the plant, such as pectin or cellulose. For pomegranate peel, investigations on bound phenols are conducted by a few studies only with contradictory results (Dadwal *et al.*, 2017; García-Villalba *et al.*, 2015; Sun *et al.*, 2021). Moreover, it was difficult to compare and evaluate data because the applied extraction processes used different solvents and fractionation procedures.

In this scenario, the present study aimed to propose a simple and applicable hydrolytic procedure using bicarbonate to partially hydrolyze native pomegranate tannins and enrich extracts in EA. Sodium bicarbonate ($NaHCO_3$) was selected as a suitable chemical agent to develop extraction tests aimed at minimizing concentration of the salt by increasing the amount of smaller phenols and suitable to evaluate bound phenols in the peel. Acid hydrolysis (AH) by hydrochloric acid (HCl) was also applied but only for comparative purposes. The impact of varying experimental conditions on the formation and/or degradation of phenols over time was evaluated by high-performance liquid chromatography–diode array detection–mass spectrometry (HPLC-DAD-MS).

Material and Methods

Chemicals and reagents

Food-grade $NaHCO_3$ was purchased from local supermarket (Florence, Italy). Other reagents and solvents (analytical or HPLC grade) were acquired from Merck (Saint Louis, MO, USA). α+β-Punicalagin (purity ≥ 91%) was acquired from Phytolab (Vestenbergsgreuth, Germany); GA (purity ≥ 99%) and EA (purity ≥ 95%) were acquired from Extrasynthese S.A. (Lyon, Nord-Genay, France).

Plant material

Pomegranate (*Punica granatum* L.) peel samples of Wonderful and G1 varieties were purchased from Azienda Agricola Onori Maria Rosaria (Fermo, Marche, Italy) and Rio del Sol soc. Agricola (Faenza, Emilia Romagna, Italy). Peel samples were air-dried at 42°C for 3 days, cut into raw pieces, and packed into zip-lock bag until extraction trials. Before extraction, the dried peel samples were crunched using a blender (IKA® M20 Universal Mill, Staufen Germany) and filtered through a 60-mesh sieve to obtain a fine and homogeneous powder. The peel samples and their extracts used are listed in Table 1. Preliminary tests to determine the best hydrolytic conditions were performed on G20M variety samples; the chosen methodological parameters were then applied to treat four peel samples.

Decoction

Conventional extraction, called decoction, was used for comparison with hydrolyzed extracts, using a published method (Khatib *et al.*, 2017) with some modifications. Briefly, 2.5 g of dried peel were dissolved in 50-mL ultrapure water and heated at 95°C for 1 h with continuous stirring, using AREX-6 Digital PRO (Velp Scientifica SRL, Usmate, Italy) heating-magnetic stirring plate. Pomegranate tannins are non-thermolabile at the decoction temperature (Khatib *et al.*, 2017). The solution was

Table 1. List of the analyzed pomegranate peel samples and applied extraction methods.

Variety	Origin (year)	Decoction	Acid hydrolysis (AH)	$NaHCO_3$ (0.3%)	$NaHCO_3$ (0.6%)
Wonderful	Marche (2020)	W20M-D	W20M-A	W20M-B1	W20M-B2
G1	Marche (2020)	G20M-D	G20M-A	G20M-B1	G20M-B2
Wonderful	Emilia-Romagna (2021)	W21ER-D	W21ER-A	W21ER-B1	W21ER-B2
Wonderful	Emilia-Romagna (2022)	W22ER-D	W22ER-A	W22ER-B1	W22ER-B2

Notes: W: Wonderful; M: origin from Marche region (Italy); E: origin from Emilia Romagna region (Italy); D: extract from decoction; A: extraction in acidic medium; B1: extract with bicarbonate 0.3%; B2: extract with bicarbonate 0.6%.

centrifugated (Z323 K high-speed centrifuge with cooling system, Hermle Labor Technik GmbH, Germany) for 10 min at 5,000 rpm and room temperature; the supernatant was recovered and used for HPLC-DAD-MS analysis.

Extraction with hydrochloric acid

Acid hydrolysis was performed directly on the peel without any previous extraction by applying a published method (García-Villalba *et al.*, 2015), as follows: peel powder (0.05 g) was placed in 5 mL of 4-M HCl. The sample was vortexed for 1 min and placed in oven at 90°C for 1, 4, 8, and 24 h; then, it was cooled down at room temperature, adjusting pH to 2.5–3.5 with 4-M NaOH, values suitable for the chromatographic column used for HPLC analysis. The formed precipitate (pellet) was removed by centrifugation (10 min, 3,500 rpm), and the supernatant was analyzed by HPLC-DAD-MS.

The pellets were washed, testing two types of solvents: (i) 10-mL dimethyl sulfoxide–methyl alcohol (DMSO–MeOH), 50:50 (v/v) as proposed previously (García-Villalba *et al.*, 2015); (ii) 10-mL ethyl alcohol–water (EtOH–H_2O), 80:20 (v/v). After dissolution, the sample was vortexed for 2 min, then centrifuged (5,000 rpm, 10 min) and the supernatant was analyzed by HPLC-DAD-MS.

Bicarbonate-assisted extraction

Bicarbonate-assisted extraction is a laboratory-developed method conducted as described: peel powder (2.5 g) was added to 50 mL of either 0.3% (w/v) or 0.6% (w/v) $NaHCO_3$ aqueous solution; the two concentrations were chosen after preliminary extraction tests using concentration of $NaHCO_3$ ranging 0.1–3.3%. The sample was maintained under stirring on the heating plate for 60 or 120 min at an extraction temperature of 95°C. It was then centrifuged (5,000 rpm for 5 min) and supernatant was recovered. Only for HPLC analysis, the supernatant was cooled down and acidified to pH 2.5–3.5 with HCOOH, causing formation of a precipitate, which was separated after a second centrifugation (5,000 rpm, 10 min), thus obtaining second supernatant (i.e., the "extract" sample) and a precipitate. The latter precipitate was washed with 25 mL of 80:20 (v/v) EtOH–H_2O solution to recover co-precipitating phenolic fraction; the obtained mixture was sonicated for 5 min in an ultrasonic bath (Argolab, Carpi, Italy) and centrifuged (5,000 rpm, 5 min), thus obtaining a third supernatant (i.e., the "washing" sample). "Extract" and "washing" samples were collected and analyzed through HPLC-DAD-MS.

Extraction of bound phenols

Tests on bound phenols were conducted on sample W20 M variety (Table 1) with a laboratory-developed method based on total extraction of free phenols by decoction plus different washes, and successive hydrolysis of the exhausted peels to verify the release of free phenols from polymeric matrix. The decoction was done with 2.5 g of peels in water (50 mL) at 95°C for 60 min (Khatib *et al.*, 2017); then, the residual solid was recovered after centrifugation and washed twice with 25-mL EtOH–H_2O, 80:20 (v/v) in ultrasonic bath (5 min) to recover residual free phenols and the unknown polar compound (UPC). The washed solid residue was then extracted with either HCl or $NaHCO_3$. AH was performed with 50-mL 4-M HCl (4 h, 90°C) and then pH adjusted to 2.5–3 with 4-M NaOH. The hydrolysis with $NaHCO_3$ was performed with 50-mL 0.6% $NaHCO_3$ solution (1 h, 95°C). Both solutions were centrifuged at 5,000 rpm for 10 min, the supernatants were recovered, and the extracts were analyzed by HPLC-DAD-MS. All extractions were performed in triplicate.

Yields of dry extracts from extraction with $NaHCO_3$

The yields of extracts from $NaHCO_3$ hydrolysis (chosen as the best method to obtain lower molecular weight phenols-enriched extracts) were determined as dry extract weight; 10 mL of extract (decoction or hydrolysates with $NaHCO_3$) were freeze-dried using Leybold Heraeus LYOVAC GT2, GEA Lyophil GmbH,

Düsseldorf – Germany. For the extracts with $NaHCO_3$, the net weight of dry extracts was calculated subtracting the estimated salt content from the total extracts' net weight. Tests were carried out in triplicate.

HPLC-DAD-MS analysis

HPLC-DAD analysis was performed using an Agilent HP1200 liquid chromatograph equipped with a DAD detector (Agilent Technologies, Palo Alto, CA, USA) and a core-shell Kinetex column C18 (100 × 3 mm, 2.6 µm; Phenomenex, USA) by following a published method (Cecchi *et al.*, 2023) with little modifications. Mobile phases were: (a) water/formic acid (pH 3.2), and (b) CH_3CN. The following multi-step linear solvent gradient was used: 0.1–8 min, 5–25% B (v/v); 8–18 min, 25% B; 18–20 min, 25–95% B; 20–26 min, 95% B; 26–28 min, 95–0% B; and 28–32 min, 0–5% B. Elution time was 32 min, with a 10-min post-time and a flow rate 0.4 mL/min. The ultraviolet-visible (UV–Vis) spectra ranged from 200 nm to 500 nm with chromatograms acquired at 210, 254, 280, and 370 nm. The MS experiments were performed using an Agilent HP 1260L liquid chromatograph equipped with a HP1260 (G6125B) Mass Spectrometer Detector with an API/electrospray interface (Agilent Technologies). The electrospray ionization (ESI) parameters were: nitrogen flow rate of 10.5 L/min, drying gas temperature of 350°C; nebulizer pressure of 1,811 Torr; and capillary voltage of 3,500 V. Acquisition was performed in full spectrum scan (range 100–2,000 Th). The experiments were carried out in a negative ionization mode by applying fragmentation voltages of 150 V and 180 V.

Quantitative evaluation by HPLC-DAD of phenolic content

For quantitation of phenolic compounds in extracts, three calibration curves were built using standard solutions of α+*β-punicalagin,* EA, and GA, as follows:

- Gallic acid: λ = 280 nm, five data points, linearity range 0–2.5 µg; $R^2 = 0.9979$. This curve was used to quantify GA and UPC from AH, since the compound presented a similar UV spectra of GA with peak at 280 nm.
- Ellagic acid: λ = 350 nm, six data points, linearity range 0–4 µg; $R^2 = 0.9994$. This curve was used to quantify EA and its derivatives.
- Punicalagin: λ = 350 nm, five data points, linearity range 0–11 µg; $R^2 = 0.999$. This curve was used to quantify punicalagin and its derivatives.

Results were expressed in mg/g of dry matter (DM) (i.e., dried peel or dried extract [DE]).

Statistical evaluation

Each experiment was performed in triplicate and results were expressed as mean ± SD using the EXCEL software (version 2013) in-house routines. Statistical significance of the quantitative data was assessed with one-way ANOVA and *F*-test at $p < 0.05$, which were performed using the EXCEL software (version 2013). The least significant difference (LSD) procedure was used as a *post hoc* comparison (DSAASTAT software v.1.1, Onofri, Pisa, 2007). Two-way ANOVA was carried out with the Origin Software, version 2024b (Northampton, MA, USA) to assess significance at $p < 0.05$.

Results and Discussion

Evaluation of phenols recovered after acid hydrolysis

The AH of pomegranate peel samples with hydrochloric acid was used for comparative purposes by applying a previous analytical protocol (García-Villalba *et al.*, 2015). The type and concentration of hydrochloric acid and the process temperature were the same as already reported (4-M HCl at 90°C), while variations in extraction time and washing method of the solid residue after extraction were applied. Figure 1 shows the main changes that occurred from 1 to 24 h for the G20M sample selected to perform a preliminary screening focused on choosing the best extraction time.

The first difference from the reference study (García-Villalba *et al.*, 2015) was the presence of an unknown polar compound (UPC, Figure 1A) in all samples, not detected previously. The molecule showed a retention time of only 2.95 min on a reverse phase column and a maximum absorption at 280 nm; it achieved maximum amount at 8 h and was not found either in decoction or after extraction with bicarbonate. UPC was not recognized as a derivative of the hydrolysis of pomegranate tannins because its chemical features did not confirm the presence of a phenolic structure (see Supplementary Material). It did not respond to electrospray ionization either in negative or positive mode, not even applying different fragmentation energies, and the proton spectrum did not show signals attributable to phenolic rings. Further studies are underway to define the structure of UPC and its precursors.

The phenolic profiles of G20M sample at different times of AH are reported in Figure 1B, along with the decoction profile as a reference. Punicalagin concentrations presented a strong decrease after 1 h of hydrolysis and disappeared at longer extraction times. Simultaneously, the concentration of punicalin increased significantly, compared to decoction after 1 h of hydrolysis because

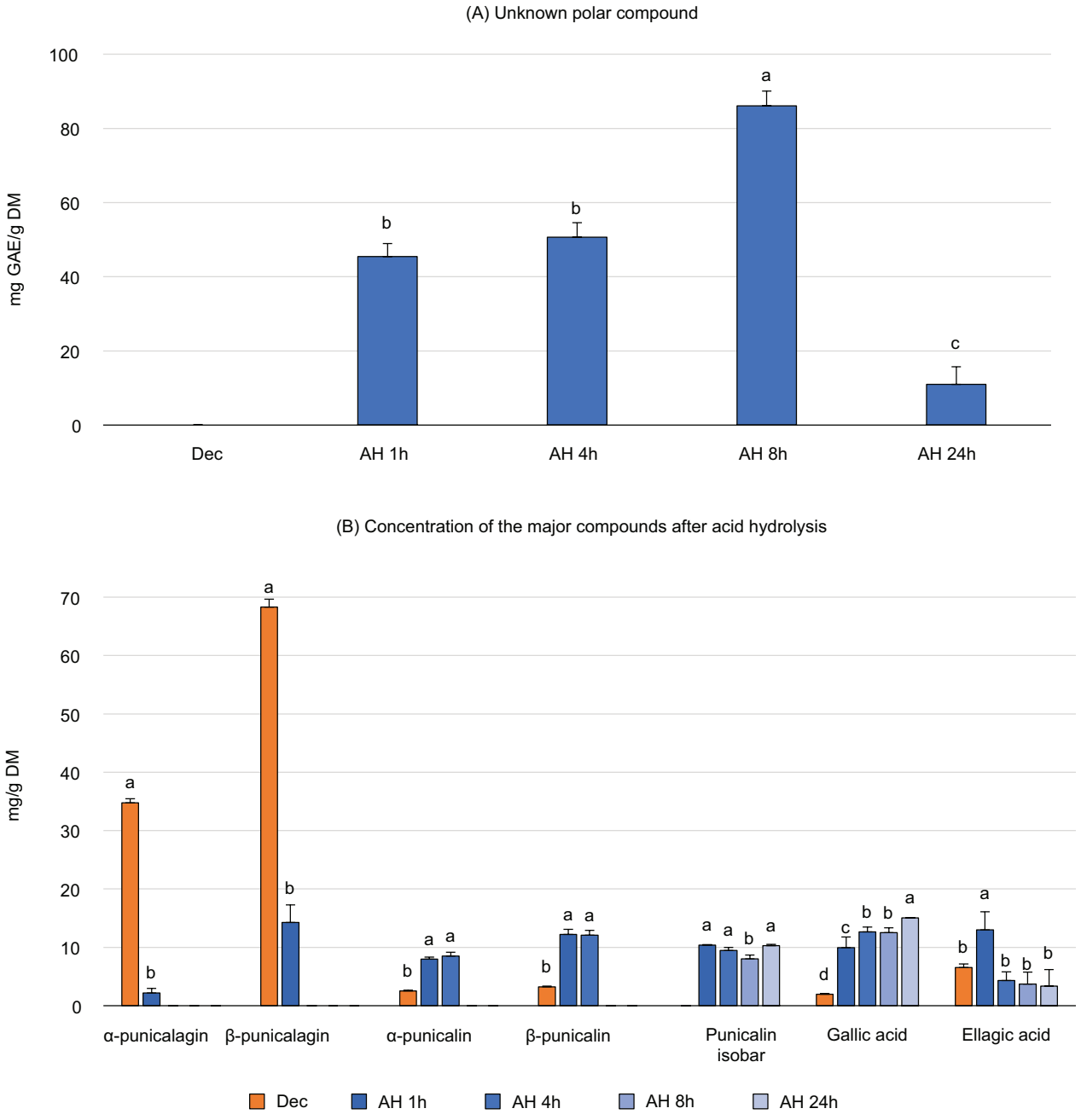


Figure 1. Distribution of UPC and main phenolic molecules in decoction extracts and acid hydrolysates of pomegranate peel G20M after different extraction times. The concentration of UPC is expressed in mg GAE/g DM and the concentration of main phenols in mg/g of DM. For each molecule, different alphabets for different treatments indicate significant differences at $p < 0.05$. Dec: decoction; UPC: unknown polar compound; GAE: gallic acid equivalent; DM: dried matter.

punicalagin lost hexahydroxydiphenoyl (HHDP) moiety. For 1 to 4 h, punicalins were stable, likely because of an equilibrium between the continuous release determined by the hydrolysis of precursors and their further degradation; after a prolonged time of hydrolysis (8–24 h), both punicalagin and punicalin disappeared. These data are partially in contrast with those reported previously by García-Villalba *et al.* (2015), which found punicalins up to 12 h of acid extraction. Compared to decoction, EA was significantly increased in 1-h acid hydrolysate but decreased at longer hydrolysis times because of low solubility in acidic water (Bala *et al.*, 2006).

Formation of a precipitate and its composition are discussed in Section 3.3. After the screening of G20M sample, extraction of 4 h was applied on Wonderful samples

to investigate any phenolic variations at the time of punicalin degradation. Extraction time of 8 h was also applied because of the major recovery of interesting compounds in washing samples (Table S1; Section 3.3), allowing observations of differences among water extracts and the insoluble phenolic fraction. Figure 2 compares the kinetics of phenolic compounds in the water extracts obtained from the Wonderful peel samples after 4- and 8-h hydrolysis with HCl.

The trend observed for phenols in sample G20M (Table S1) was also confirmed for other Wonderful samples. In addition, in this case, UPC was produced, reaching its maximum concentration after 8 h.

Punicalin isobar was one of the main phenols in aqueous extracts and it increased in all samples after 4 h (Figure 2); a different behavior among peel samples was observed at 8 h, because only for W21ER and W22ER samples, it increased further, but a slight decrease was observed for other samples. Such different behaviors are attributable to differences in peel composition. Concerning α- and β-punicalins, all samples showed the same trend with a maximum increase after 4 h, while prolonged reaction time determined almost complete degradation. In agreement with the data shown in Figure 1B and the poor solubility of EA in acidic water, it partially precipitated determining low concentration in extracts and a high variability among replicates, not observed for other

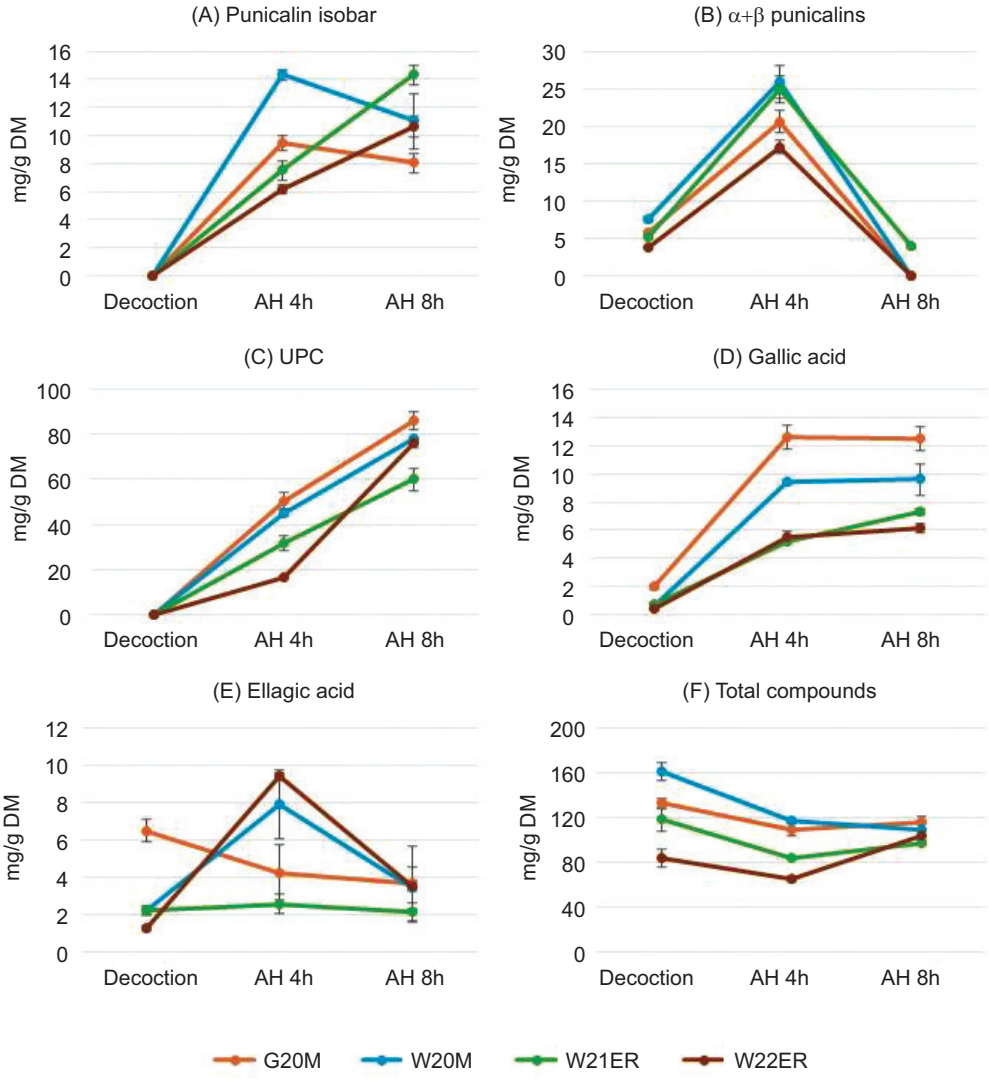


Figure 2. Kinetics over time (0, 4, 8 h) of UPC and main phenols in the acidic extracts of four peel samples and decoction as reference sample for time 0. Data are mean values of triplicate performance, expressed as mg/g of DM. According to Table 3, α+β-punicalins are compounds 9 and 10 and punicalin derivative is compound 2. Regarding total compounds, the sum includes all minor phenols detected in the extracts.

molecules. Total compounds, which as a sum include all minor phenols detected in the extracts, resulted lower if compared to the decoction (Figure 2F), mainly because lipophilic molecules, such as EA and GA released by the degradation of punicalagins and punicalins, partially precipitate in the acidic environment. A slight increase for some samples after 8 h of hydrolysis was observed because UPC was also considered. Overall, the acidic extraction proposed previously (García-Villalba *et al.*, 2015) to hydrolyze the tannins of pomegranate showed several critical issues, such as a strong degradation of native phenols and use of HCl as a chemical agent, thus appearing unsuitable for future scale-up processes applied to pomegranate peels.

Evaluation of phenols after hydrolysis with $NaHCO_3$

Concerning extraction of pomegranate peel with bicarbonate solutions, preliminary tests with variable $NaHCO_3$ concentrations (0.1–3.3% w/v) and reaction time (20–120 min) were performed to select suitable salt concentrations and process time. A complete degradation of tannins occurred when exceeding 1% (w/v) of bicarbonate with a reaction time of 60 min or more, and similar results were obtained with a reaction time of 20 min and a bicarbonate concentration of 3.3% (data not shown). Along with the reaction time of 60 and 120 min, lower concentrations of bicarbonate (0.3% and 0.6%) were successively selected to reduce final salt content in the extract. Further tests were conducted with G20M sample within the defined experimental ranges. The distribution of main molecules after hydrolysis with 0.3% w/v $NaHCO_3$ (B1) and 0.6% w/v $NaHCO_3$ (B2) for 60 and 120 min, compared with decoction (D), are reported in Figure 3.

For both concentrations, a partial hydrolysis of original tannins was observed with a higher degree of hydrolysis for B2 samples. A higher degradation of β-punicalagin and α-punicalin was reached in B2 samples (B2, 60 min). Furthermore, EA showed the same concentration in both B1 and B2 samples after 60 min. Overall, significant decreases of α- and β-punicalagin were observed in all samples, compared to decoction. Considering the results in Figure 3, the extraction time of 60 min and the bicarbonate concentrations of 0.3% and 0.6% w/v were selected to treat all peel samples listed in Table 1.

As reported in Figure 4, GA and EA were the main hydrolysis products in all samples, their content increased proportionally with the increase of bicarbonate, with a similar trend for α- and β-punicalin.

Differently, α- and β-punicalagin, the main precursors of previous molecules, decreased proportionally with the

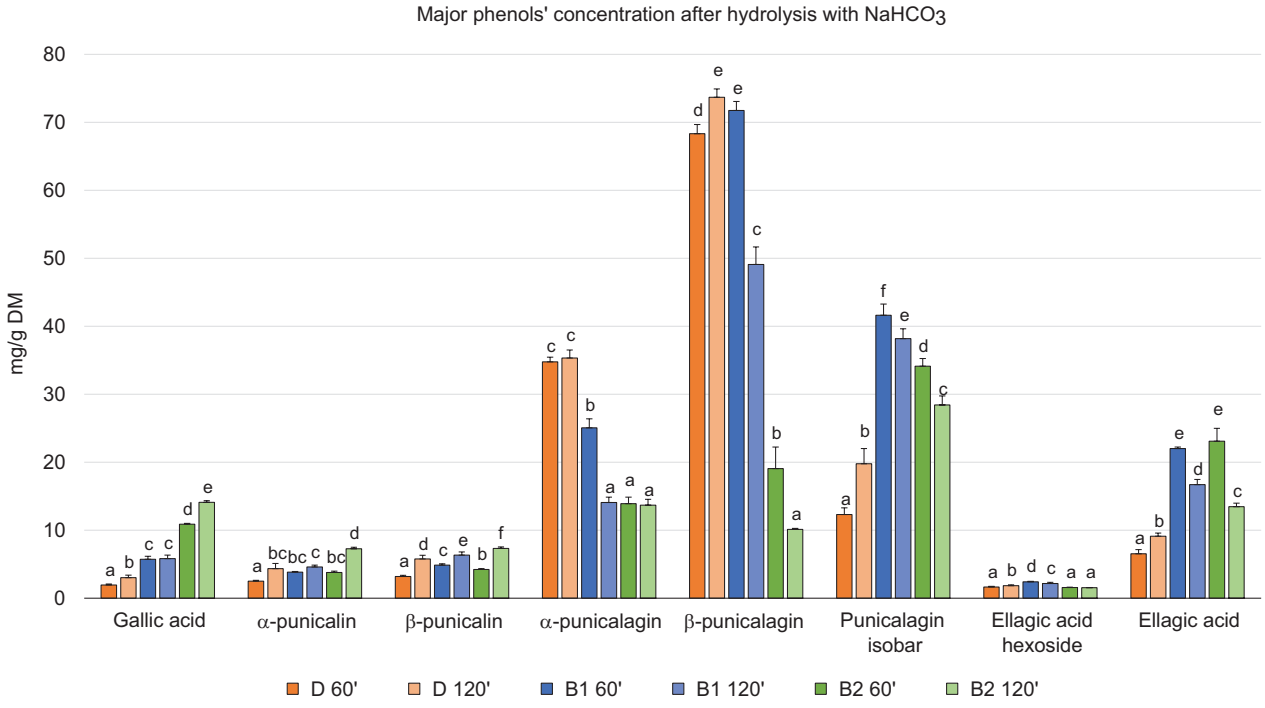


Figure 3. Distribution of major phenolic molecules in extracts from the G20M sample after different times of extraction with bicarbonate at two concentrations. Data are expressed as mg/g DM and different alphabets for different treatments indicate significant differences at $p < 0.05$. D: decoction; B1: extraction with 0.3% $NaHCO_3$; B2: extraction with 0.6% $NaHCO_3$.

increase of bicarbonate. It is worth highlighting that the presence of punicalagin isobars (compounds 12 and 13 in Table 3) which increased with the same kinetics of EA, results in major compounds in bicarbonate extracts. Their origin could be hydrolysis and rearrangement of one ester bond of precursor molecules (α- or β-punicalagin). Overall, the data shown in Figure 4 highlighted that a partial hydrolysis of native tannins was obtained in non-alkaline environment, because the sample solution from $NaHCO_3$ at 0.3% was associated with pH values close to neutrality, suggesting a possible catalytic effect of bicarbonate in the hydrolysis of punicalagin's ester bonds, not associated with an alkaline environment. Finally, among the extracts of the same peel sample, the total phenolic amount of the supernatant, which included all minor phenols, did not show significant variations from decoction to B1 and B2 samples (Figure 4F). In light of the data in Figure 4, extraction with 0.6% bicarbonate for 1 h could be proposed as a simple method to increase the amount of low molecular weight phenols, including EA and to reduce the concentration of punicalagins, without reducing total amounts. Further discussion on total phenols is in presented Sections 3.4 and 3.5.

Phenols after hydrolysis recovered in washing samples

Since part of the released phenols during hydrolysis are lipophilic molecules, with low water solubility, a washing step was applied to solid residues formed after AH

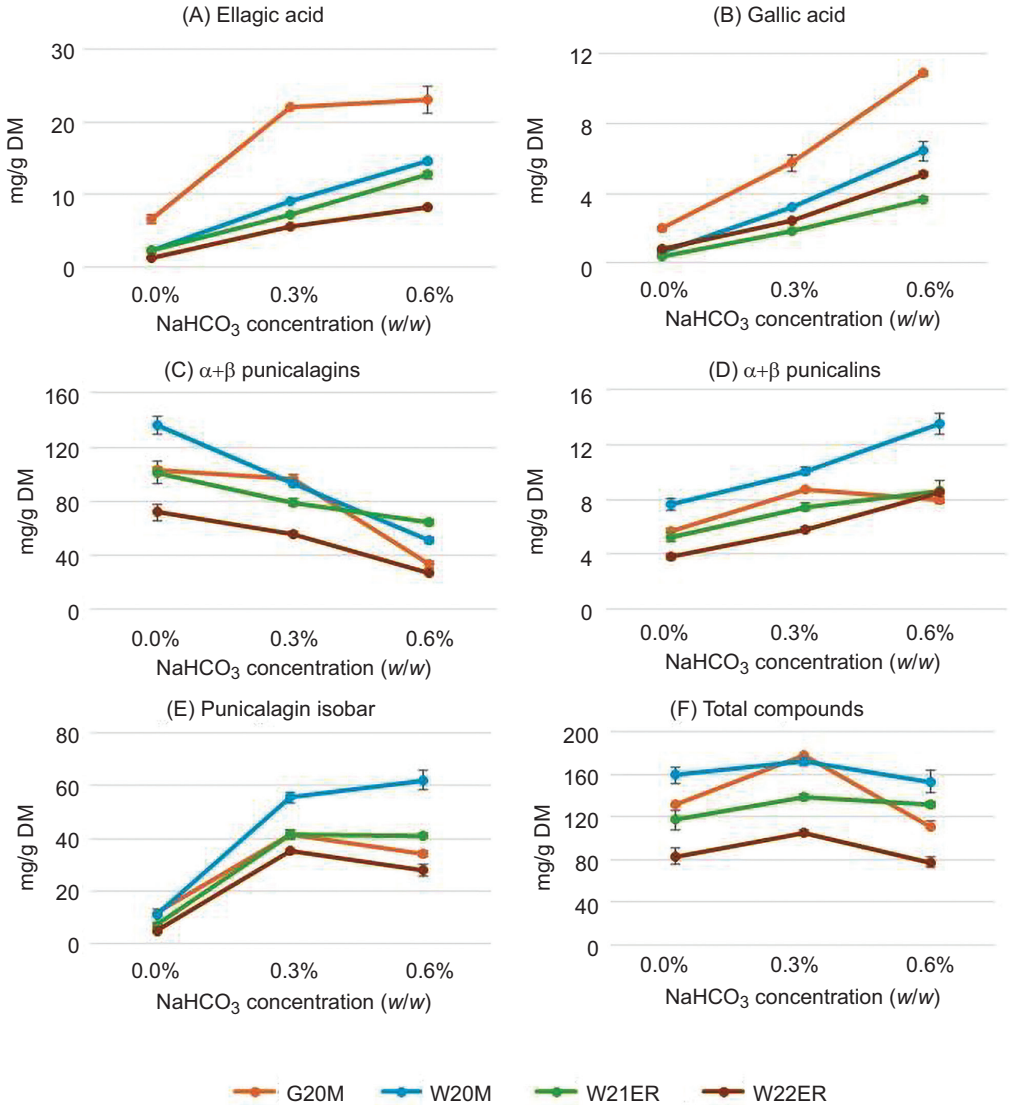


Figure 4. Amount of different phenols in the extracts from $NaHCO_3$ obtained from four different samples of peel after 60 min of extraction time. The data are expressed as a mean value of triplicates and as mg/g of DM; 0% of $NaHCO_3$ indicates the extract obtained after decoction. Regarding total compounds (F), the sum includes minor phenols detected in extracts.

and for the precipitates formed after the acidification of bicarbonate extracts before the HPLC analysis of supernatant. In particular for EA, its planar structure confers a high degree of crystallinity and a poor solubility in water with a maximum of 9.7 μg/mL at neutral or acidic pH (Bala *et al.*, 2006). To accurately estimate EA and other lipophilic phenols released after hydrolysis, the washing samples were evaluated by HPLC-DAD (results presented in Tables 2A, 2B, and S1).

Concerning acid extraction, two washing mixtures were tested: DMSO–MeOH, 1:1 v/v, as an ideal solvent for good recovery of water-insoluble phenols as indicated in the literature (García-Villalba *et al.*, 2015), although it hinders the drying process because of high boiling point and is not of food-grade nor applicable for possible scale-up processes; EtOH 80% v/v was selected because it was of food grade, certainly usable for a scale-up, and easily removable, thus enabling the acquiring of dry extracts.

The effectiveness of two different solvents in recovering insoluble phenols from the solid residues of G20M and W20M samples was evaluated preliminarily (Table S1). The washing samples were characterized by the presence of valoneic acid dilactone, gallagic acid dilactone, and EA. Regarding recovered phenols, DMSO–MeOH solution was more exhaustive than EtOH 80% solution, giving approximately a double amount of total phenols, with EA being by far the most abundant compound. Hydroalcoholic solvent was, however, suitable for recovering a good part of precipitated phenols and was therefore applied to other peel samples of the Wonderful variety: two major compounds significantly changed during acidic hydrolysis, namely valoneic acid and EA, while total phenols differed among samples at different times, and significantly increased at 8 h in all washing samples (Table 2A).

Concerning the extraction with bicarbonate, the formation of a precipitate was only observed at the end of extractive steps, during the pH adjustment with formic acid required for HPLC analysis. Consequently, such precipitate cannot be considered a residue of the one-step extraction but a fraction of the whole aqueous extract from bicarbonate. Indeed, the 80% EtOH-wash samples showed similar profiles as those observed for the corresponding extract (supernatant) with only increased amounts of EA, especially in the samples extracted with 0.6% $NaHCO_3$ (Table 2B). The significance of independent variables (sample and time for AH and sample and bicarbonate concentrations for hydrolysis with alkaline agent) and their interaction according to 2-way ANOVA are reported in Tables S2A and S2B, respectively. The residue produced after AH (Table S2A) contained mostly valoneic acid dilactone, GA, and EA. However, time significantly affects only valoneic acid and GA. Interestingly, no overall effect of time was observed on EA content, although the interaction between sample and time was significant ($p < 0.05$). Nevertheless, the total content was significantly affected ($p < 0.001$) by both time and sample type.

Regarding the residues obtained after hydrolysis with bicarbonate (Table S2B), the levels of GA and EA were significantly affected by bicarbonate concentrations. For the native α-punicalagin and β-punicalagin, both compounds were significantly affected by sample, bicarbonate concentration, and their interaction. Similarly, the amount of new isobar of punicalagin was significantly influenced by both sample type, bicarbonate concentration, and their interaction ($p < 0.001$).

Total phenols recovered after hydrolysis

Total phenols extracted from dried peels were evaluated in the extracts of Wonderful peel samples, because this is one of the most important and widely cultivated varieties globally. Differently, the G1 variety was included in this study for different morphology of the mesocarp (much thinner than the Wonderful variety), although it is cultivated only in Italy. From the data discussed above, it was observed that this different morphology is not related to relevant variations in the mesocarp's tannin profile.

Total phenols extracted were calculated as sum of phenols recovered in both supernatant and EtOH 80% washing samples (Figure 5).

Phenols after AH were statistically comparable to those of the decoction for 8-h hydrolysates, while in the case of 4-h hydrolysates, amounts were significantly lower for all samples. B1 methodology (60 min with 0.3% bicarbonate) was the one that extracted maximum phenols for all samples, significantly higher than in all other extracts. A small decrease in B2 samples was observed due to the stronger punicalagin degradation when a greater quantity of bicarbonate was used, according to data shown in Figures 3 and 4. However, the B2 method still recovered slightly higher amounts, compared to decoction when the residue was considered.

Yields and total phenolic content (TPC) in dried extracts

Considering the high content of phenols extracted through bicarbonate and the possibility to use these samples for future biological tests also due to the increased content of low-molecular weight phenols, including EA, the percentage yields of the dried extracts of dry peels and TPC (mg of phenols/g of DE) were determined.

Table 2. Phenols recovered from pomegranate peel of the Wonderful variety after a washing step with EtOH–H_2O, 80:20 (v/v) of (A) the solid residue after acidic hydrolysis (AH), and (B) the precipitate after hydrolysis with bicarbonate and successive acidification.

Compound	Samples	4 h	8 h
(A) Phenols in solid residue after AH (mg/g DM).			
UPC	W20M	2.86 ± 0.88^{c,d}	7.74 ± 0.73^{a}
	W21ER	1.80 ± 0.50^{d,e}	5.92 ± 0.13^{b}
	W22ER	0.86 ± 0.27^{e}	5.96 ± 0.04^{b}
Ellagic acid-hexoside isomer 1	W20M	2.13 ± 0.17^{a}	n.d.
	W21ER	0.89 ± 0.08^{c,d}	0.99 ± 0.08^{b,c}
	W22ER	0.79 ± 0.01^{d}	n.d.
Valoneic acid dilactone	W20M	3.50 ± 0.19^{c}	5.26 ± 0.60^{b}
	W21ER	1.66 ± 0.14^{e}	3.00 ± 0.28^{d}
	W22ER	1.96 ± 0.12^{e}	2.06 ± 0.09^{e}
Gallagic acid	W20M	3.85 ± 0.25^{b,c}	6.82 ± 3.93^{a}
	W21ER	0.70 ± 0.11^{e}	3.30 ± 0.79^{c,d}
	W22ER	0.90 ± 0.11^{d,e}	3.09 ± 0.16$^{c–e}$
Ellagic acid-hexoside isomer 2	W20M	1.52 ± 0.09^{a}	1.50 ± 0.56^{a}
	W21ER	0.83 ± 0.03^{c,d}	1.47 ± 0.04^{a}
	W22ER	0.51 ± 0.01^{d}	0.59 ± 0.01^{d}
Ellagic acid	W20M	21.9 ± 0.8^{a,b}	25.8 ± 3.4^{a,b}
	W21ER	11.6 ± 0.3^{c}	22.1 ± 6.5^{a,b}
	W22ER	16.4 ± 0.3^{b,c}	12.3 ± 0.6^{c}
Total content	W20M	35.7 ± 0.8^{b}	49.6 ± 2.0^{a}
	W21ER	17.5 ± 0.7^{d}	36.8 ± 7.2^{b}
	W22ER	21.5 ± 0.3^{c,d}	24.0 ± 0.8^{c}

Notes: Data (expressed as mg/g of dry matter [DM]) of main compounds are mean ± SD in triplicate. For each molecule, different superscript letters indicate significant differences at $p < 0.05$ according to 2-way ANOVA.
n.d.: not detected; UPC: unknown polar compound.

Compound	Samples	0.3% (60 min) EtOH–H_2O (80:20)	0.6% (60 min) EtOH–H_2O (80:20)
(B) Phenols precipitated after bicarbonate hydrolysis and successive acidification (mg/g DM).			
Gallic acid	W20M	0.7 ± 0.1^{b,c}	0.7 ± 0.1^{c}
	W21ER	0.5 ± 0.1^{d}	0.8 ± 0.1^{a,b}
	W22ER	0.5 ± 0.1^{d}	0.9 ± 0.1^{a}
α-punicalin	W20M	1.2 ± 0.2^{b}	1.7 ± 0.1^{a}
	W21ER	0.5 ± 0.2^{d}	0.4 ± 0.1^{d}
	W22ER	0.7 ± 0.1^{c}	1.1 ± 0.1^{b}
β-punicalin	W20M	1.4 ± 0.3^{a}	1.8 ± 0.1^{b}
	W21ER	1.3 ± 0.2^{b,c}	1.1 ± 0.1^{c}
	W22ER	0.8 ± 0.1^{d}	1.2 ± 0.1^{c,d}
Punicalagin isobar	W20M	11.2 ± 2.1^{a}	13.1 ± 1.2^{a}
	W21ER	11.6 ± 1.8^{a}	2.7 ± 0.3^{c}
	W22ER	8.4 ± 0.3^{b}	6.3 ± 0.8^{b}

(*continues*)

Table 2. Continued.

Compound	Samples	0.3% (60 min) EtOH–H_2O (80:20)	0.6% (60 min) EtOH–H_2O (80:20)
α-punicalagin	W20M	11.1 ± 2.1^{a}	5.6 ± 0.4^{c,d}
	W21ER	8.6 ± 1.2^{b}	5.3 ± 0.3^{d}
	W22ER	7.3 ± 0.2^{b,c}	3.5 ± 0.1^{e}
β-punicalagin	W20M	8.6 ± 1.4^{b}	6.9 ± 0.4^{c}
	W21ER	10.9 ± 1.6^{a}	6.9 ± 0.4^{c}
	W22ER	6.5 ± 0.3^{c}	4.6 ± 0.1^{d}
Ellagic acid hexoside	W20M	0.4 ± 0.1^{c,d}	0.6 ± 0.1^{a,b}
	W21ER	0.5 ± 0.2^{b,c}	0.7 ± 0.1^{a}
	W22ER	0.3 ± 0.1^{d}	0.4 ± 0.1^{c,d}
Ellagic acid	W20M	2.5 ± 0.4^{d}	6.6 ± 0.4^{a}
	W21ER	3.1 ± 0.4^{c}	4.7 ± 0.3^{b}
	W22ER	2.4 ± 0.1^{d}	5.3 ± 0.4^{b}
Total content	W20M	37.1 ± 6.7^{a}	37.0 ± 2.7^{a}
	W21ER	36.9 ± 5.6^{a}	22.7 ± 1.6^{b}
	W22ER	26.9 ± 0.9^{b}	23.1 ± 1.5^{b}

Notes: Data (expressed as mg/g of DM) of main compounds are mean ± SD in triplicate. For each molecule, different superscript letters indicate significant differences at $p < 0.05$ according to 2-way ANOVA.

Because the bicarbonate extraction protocol required the addition of sodium salt and subsequent acidification with formic acid prior to HPLC analysis, sodium formate was present in liquid extract estimated at 2.1 mg/mL and 4.3 mg/mL for 0.3% and 0.6% bicarbonate, respectively. The corresponding amounts were subtracted from the total weight of each dry extract for obtaining net percentage yields of dried peel (Figure 6A).

For each sample, the mean net yields increased proportionally to the bicarbonate concentration, reaching a maximum of about 61% for G20M variety sample in the case of 0.6% of bicarbonate. The yields obtained from decoction were always lower, ranging 43–53%, with changes depending on the origin of peel sample. It was supposed that the use of bicarbonate facilitated the release of further polar compounds from the peel, compared to decoction.

The TPC was consequently evaluated as mg/g of DE (Figure 6B). The highest TPC was found in the B1 extracts of all samples with a range of 241.0–361.3 mg/g DE. In B2 samples, TPC was always significantly lower than decoction and B1 samples (172.9–285.9 mg/g DE), although with slight differences. The differences in tannin content among samples collected over three different years (Figure 6B) resulted from significant variations in levels of tannin expressed on dry peel and were consistent with the data shown in Figure 4F. Variations in tannin richness among pomegranate peel samples, even within the same variety, is attributed to geographic origin, climatic conditions, and age of tree, with older plants producing lower levels of phenolic compounds (Dadáková *et al.*, 2020). Within each peel sample, the use of bicarbonate allowed producing final dry extracts with increased or comparable amounts of TPC, compared to a reference sample, such as decoction, but otherwise characterized by higher amounts of low-molecular weight phenols.

Evaluation of bound phenols

Hydrolytic procedures, such as acid or alkaline hydrolysis, are often applied to evaluate the amount of bound phenols in different vegetal or fruits (Zhang *et al.*, 2020). The presence of bound phenols in pomegranate peels is scantly investigated in the literature (Dadwal *et al.*, 2017; García-Villalba *et al.*, 2015; Sun *et al.*, 2021). The authors of these studies, although applying different extraction procedures, observed an increase in phenolic content after hydrolysis, suggesting that a part of phenols in pomegranate peel is covalently bound to polymeric structures (e.g., through ester bonds to cellulose and pectin).

In this study, to prove the presence of bound phenols in pomegranate peel and to determine their content, decoction for 60 min was selected as a conventional extraction. The peels of W20M variety sample were first extracted by decoction (D), then the solid residue was submitted to two washing steps (Wash 1 and Wash 2) to remove any residual phenols, and finally the solid residue was hydrolyzed with HCl (acid hydrolysis) or bicarbonate at 0.6% w/v (B2). As

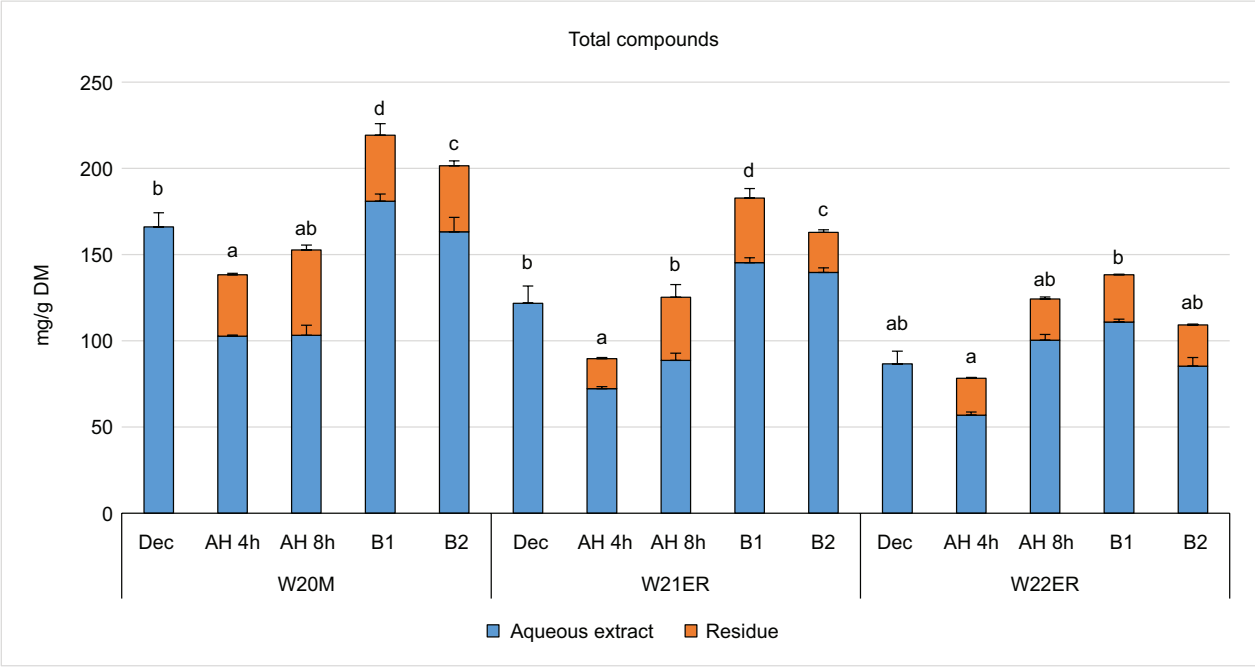


Figure 5. Total phenols extracted from Wonderful variety samples, reported as the sum of phenols in supernatant (light blue) and washing of solid residues/precipitates (orange), and expressed as mg/g of DM. Dec: decoction; AH: samples in acidic media; B1 and B2: samples from extraction with bicarbonate at 0.3% and 0.6%, respectively; DM: dry matter. Different alphabets indicate significant differences at $p < 0.05$.

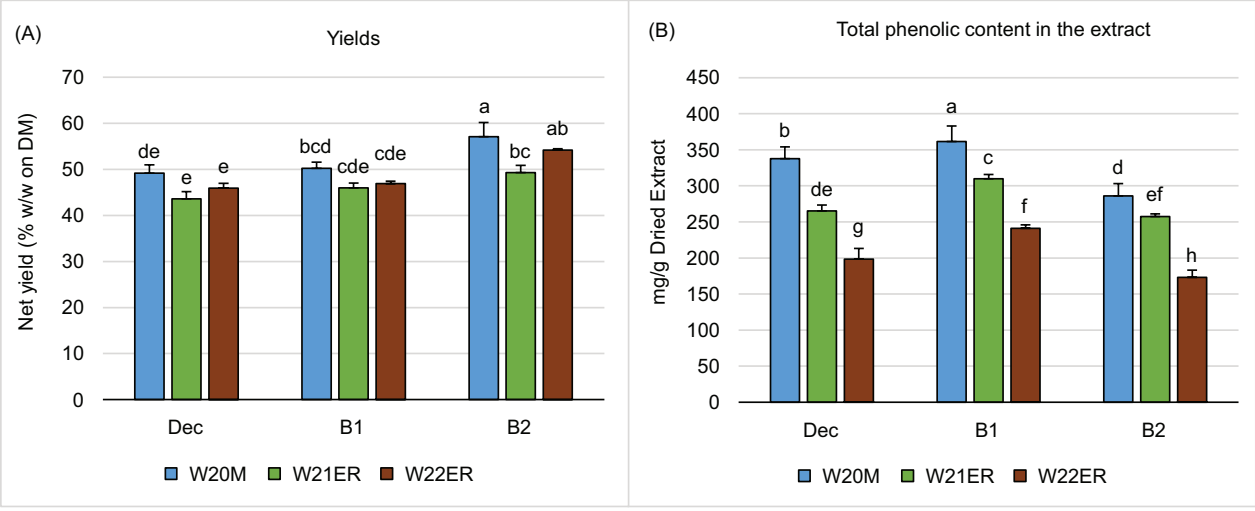


Figure 6. (A) Yields of dry extracts after extraction with bicarbonate, expressed as percentage of dried extract on dried peels; (B) total phenolic content (TPC) in the extract, expressed as mg/g of direct extract.. Dec: decoction; B1 and B2: from extraction with bicarbonate at 0.3% and 0.6%, respectively; DM: dried matter. Different alphabets indicate significant differences at $p < 0.05$.

shown in Table S3, Wash 1 contained approximately 10% of phenols extracted in hot water and only 1.2% of phenols were in Wash 2 sample, while after acidic hydrolysis of the washed residue, only UPC was detected (data not shown) and no other phenolic compound was revealed. The extract with 0.6% of $NaHCO_3$ contained 3.7% of total phenols (around 6 mg/g of DM), with 2.8 mg/g of EA and limited amounts of punicalins and punicalagins (from 0.3 to 1.3 mg/g DM). Based only on alkaline hydrolysis, the presence of bound phenols in pomegranate peels was confirmed, but in very low amount.

Bound phenols in other fruits (e.g., cranberries, strawberries, red grapes, grapefruits, apples, pineapples,

pears, bananas, peaches, lemons, and oranges) showed a similar content, ranging 2–24% of total phenols in common fruit (Sun *et al.*, 2002). Additionally, the measured phenols released after mild alkaline hydrolysis included mainly ellagitannin derivatives, known to be little water soluble, leading to possible underestimation. Literature studies estimated the content in bound phenol on pomegranate peels of 50% (Dadwal *et al.*, 2017). Nevertheless, in the latter case, because the measurements were done by a colorimetric assay, an overestimation of real content was hypothesized, as already observed for chestnut tannins passing from HPLC to Folin–Ciocalteu results (Khatib *et al.*, 2023). A different study that discussed bound phenols in pomegranate peels found few isomers of punicalagins along with valoneic acid; however, no quantitative evaluation was carried out. In this case, the main detected compounds were glycosides, sterols, and pentacyclic triterpenoids compounds (Sun *et al.*, 2021), whose recovery was mostly driven by the non-polar nature of the solvent used for suspending the exhausted residue of peels.

Bound phenols or non-extractable phenols were estimated in 11 pomegranate peel samples (without specifying varieties), assessing that after acidic hydrolysis it was possible to recover up to 36% more phenols (non-extractable fraction) than by conventional extraction (García-Villalba *et al.*, 2015). In our study, only 3.7% of non-extractable phenols were found in the Wonderful variety (Table S4) by not applying the acid extraction but the extraction with bicarbonate 0.6%. Differently from our study, the study conducted by García-Villalba *et al.* (2015) used conventional method for extractable (or free) ellagitannins by a single-step extraction at room temperature with 0.1% of HCl in MeOH/DMSO/H_2O (4:4:2, v/v/v), with 10 min of extraction time. No other extraction or washing was successively applied to solid residue to verify whether the extraction was exhaustive.

Such a large difference is mainly related to different extraction procedures applied to evaluate free or extractable tannins. In this research, different reasons were indicated to use non-acidified hot water as the best extraction mixture to recover free ellagitannins: (i) ellagitannins are well-soluble in this medium and stable at 100°C; (ii) the hot treatment is suitable to better disaggregate the structure of peel and favors the extractability of phenols; and (iii) hot extraction for 60 min was proven to be exhaustive (Khatib *et al.*, 2017).

Although our data revealed a very low amount of bound phenols in pomegranate peel from the Wonderful variety, further research, including a larger number of samples, is needed to confirm this result for the peels of other less studied pomegranate varieties.

Identification of phenolic compounds in hydrolyzed extracts

The complete list of phenolic compounds detected in both hydrolyzed and non-hydrolyzed extracts is given in Table 3, which shows presence/absence of the compound in decoction (reference method without hydrolysis) and extracts from hydrolysis methods. The compounds were tentatively identified by the UV-Vis spectra, retention time, comparison with data from the literature (Akande *et al.*, 2022; Gu *et al.*, 2013; Hemingway and Hills, 1971; Hernández-Corroto *et al.*, 2019; Man *et al.*, 2022; Plumb *et al.*, 2002; Sentandreu *et al.*, 2013; Tanaka *et al.*, 1990; Tuominen and Sundman, 2013) and by the MS fragmentation pattern in negative ionization mode.

Compound 2 with molecular ion at mass-to-charge ratio (m/z) of 781 was identified as an isobar of α-punicalin and β-punicalin with a lower retention time. This hypothesis is explained by the possible rearrangement of a punicalin intramolecular bond to reach a more stable structure in acidic environment. In fact, this isobar was detected in acidic hydrolysates after longer extraction time (8 and 24 h) where precursor punicalins were completely degraded.

Compounds 3 and 4 were tentatively identified as HHDP-glucose isomers, mainly because their mass spectra showed molecular ions at 481 m/z, typical of this structure. These compounds were detected in both decoction and extracts hydrolyzed with bicarbonate.

Compounds 5 and 6 were identified as GA monohexoside and GA, respectively, because of the presence of molecular ion at 331 m/z for glycoside, and at 169 m/z for GA. Identification of GA was confirmed by comparison with pure standard. Small amounts of GA were detected in decoction, and its concentration increased proportionally to the bicarbonate concentration or to the reaction time during AH (see Sections 3.1 and 3.2). GA increase during hydrolysis is explained by the degradation of many minor compounds containing galloyl residues, such as compounds 20, 30, 33, etc.

Compound 7 was tentatively identified as galloyl-HHDP-hexoside according to the molecular ion at 633 m/z (Figure 7). The molecule was detected in both decoction and extract hydrolyzed with 0.3% $NaHCO_3$ but was absent in 0.6% $NaHCO_3$ extract and in the samples obtained by AH. Also, compound 31 (rt = 7.52 min) presented the same molecular ion and similar UV-VIS spectrum, allowing its identification as an isomeric form of galloyl-HHDP-hexoside. This isomer was detected in all extracts. Applying extract ion function at 633 m/z, it was possible to find several isomers, which presented this ion in their mass spectrum as a molecular peak; however,

Table 3. Phenolic compounds of pomegranate peels tentatively identified in different extracts by HPLC-DAD-MS.

Peak	rt (min)	λ_{max} (nm)	[M-H]-	[M-2H]2-	[2M-H]-	Fragments	Identification	G20M-D	G20M-B1[a]	G20M-B2[b]	G20M-A[c]
1.	1.30	256, 378	794	-	-	560	Unknown	n.d.	n.d.	n.d.	x
2.	1.34	258, 378	781	390	-	-	Punicalin isobar	n.d.	n.d.	n.d.	x
3.	1.40	n.d.	481	-	-	-	HHDP-Glc isomer 1	x	x	x	n.d.
4.	1.48	n.d.	481	-	-	-	HHDP-Glc isomer 2	x	x	x	n.d.
5.	1.63	266	331	-	-	-	Gallic acid glucoside	x	x	x	x
6.	2.07	270	169	-	-	125	Gallic acid	x	x	xx	xx
7.	2.08	262	633	-	1,267	-	Galloyl-HHDP-hexoside	x	x	n.d.	n.d.
8.	2.29	280, 370	515	-	-	-	Unknown	x	x	x	n.d.
9.	2.45	260, 378	781	390	-	-	α-punicalin	x	x	x	x
10.	2.60	260, 378	781	390	-	-	β-punicalin	x	x	x	x
11.	2.95	228, 278	NI	-	-	-	Unknown	n.d.	n.d.	n.d.	xx
12.	3.42	258, 378	1,083	541	*1,625	-	Punicalagin isobar	x	xx	xx	n.d.
13.	3.77	258, 378	1,083	541	*1,625	-	Punicalagin isobar	x	xx	xx	n.d.
14.	5.15	258, 370	263	-	-	191	Unknown	n.d.	n.d.	n.d.	x
15.	5.53	258, 376	1,083	541	*1,625		α-punicalagin	x	x	n.d.	n.d.
16.	5.57	256, 374	469	-	-	425	Valoneic acid dilactone	x	x	x	x
17.	5.58	258, 286, 370	377	-	-	347	Unknown	n.d.	n.d.	n.d.	x
18.	5.78	260	483	-	-	-	Digalloyl-hexoside	x	x	x	n.d.
19.	5.89	260, 378	1,039	519	-	-	Punicalagin decarboxylation derivative	n.d.	x	x	n.d.
20.	6.36	260	785	392	-	1,567	Digalloyl-HHDP-hexoside	x	n.d.	n.d.	n.d.
21.	6.50	262, 375	801	400	-	-	Digalloyl-HHDP-gluconic acid	x	x	x	n.d.
22.	6.51	260, 378	1,039	519	-	-	Punicalagin decarboxylation derivative	n.d.	x	x	n.d.
23.	6.70	258, 375	1,083	541	*1,625	-	β-punicalagin	x	x	n.d.	n.d.
24.	6.76	262, 365	291	-	-	247	Brevifolin carboxylic acid	x	x	xx	x
25.	6.93	270, 372	277	-	-	-	Unknown	n.d.	n.d.	n.d.	x
26.	7.01	260	799	-	-	-	Lagerstannin A	x	n.d.	n.d.	n.d.
27.	7.10	258, 366	783	-	-	631	Pedunculagin isobar	n.d.	n.d.	n.d.	x
28.	7.14	282, 342	437	-	-	247	Gallocatechin pentoside	n.d.	x	x	n.d.
29.	7.18	258, 366	783	-	-	631, 541	Pedunculagin isobar	n.d.	n.d.	n.d.	x
30.	7.26	256	935	-		-	Galloyl-bis-HHDP-hexoside	x	n.d.	n.d.	n.d.

31.	7.52	262	633	-	1267	-	Galloyl-HHDP-hexoside	x	xx	xx	n.d.
32.	7.62	258, 366	783	-	-	633	α-pedunculagin	x	x	x	x
33.	7.75	260	785	-	-	-	Digalloyl-HHDP-hexoside	x	n.d.	n.d.	n.d.
34.	7.87	258, 368	783	-	1567	-	β-pedunculagin	x	x	x	x
35.	7.92	258, 372	1,065	532	-	463	Unknown	n.d.	n.d.	n.d.	x
36.	8.07	258, 366	1,085	542	-	-	Digalloyl-gallagyl-hexoside	x	x	n.d.	n.d.
37.	8.17	258, 308	419	-	-	-	Unknown	n.d.	n.d.	x	n.d.
38.	8.23	258, 360	1,085	542	-	-	Digalloyl-gallagyl-hexoside	x	x	n.d.	n.d.
39.	8.39	282, 368	275	-	-	-	EA decarboxylation derivative	n.d.	x	x	n.d.
40.	8.59	256, 365	469	-	-	425	Valoneic acid dilactone (isomer)	n.d.	n.d.	n.d.	x
41.	8.69	254, 360	463	-	927	301	EA-hexoside	x	x	x	x
42.	8.81	262, 360	463	-	927	301	EA-hexoside	x	x	x	x
43.	8.97	265	969	484	-	-	Granatin hydrolysis derivative	x	x	x	n.d.
44.	9.41	272	951	475	1,903	-	Granatin B	x	n.d.	n.d.	n.d.
45.	9.36	254, 380	601	-	-	-	Gallagic acid dilactone	n.d.	x	x	n.d.
46.	9.66	254, 370	463	-	927	445, 301	EA-hexoside	n.d.	n.d.	n.d.	x
47.	9.92	254, 348, 360	433	-	867	301	EA-pentoside	x	x	x	n.d.
48.	9.97	254, 368	301	-	-		Ellagic acid	x	x	x	x
49	10.81	256, 372	427	-	-	301	Unknown	n.d.	n.d.	n.d.	x
50.	11.62	256, 366	425	-	851	301	Decarboxylatedvaloneic acid dilactone	n.d.	x	x	n.d.
51.	12.36	220, 368	923	461	-	301	Unknown	n.d.	x	x	n.d.
52.	14.60	248, 355	423	-	847	-	Unknown	n.d.	x	x	n.d.

Notes: rt, retention time; n.d.: not detected; x: detected; xx: detected in concentration more than in decoction; EA: ellagic acid; HHDP: hexahydroxydiphenoyl; NI: no ionization; *hypothesized ion species: [trimeric form-2H]$^{-2}$.

[a]Hydrolysis with 0.3% $NaHCO_3$; [b]hydrolysis with 0.6% $NaHCO_3$ [c]acid hydrolysis with 4-M HCl, 4 h.

since their concentration was barely detectable, only two isomers are shown in Table 3.

Compounds 9 and 10 were identified as α- and β-anomeric forms of 4,6-gallagyl-glucoside (punicalin), as they showed $[M-H]^-$ fragment at 781 m/z and $[M-2H]^{2-}$ ion at 390 m/z. Formation of double charged ions is frequently observed in MS negative ionization mode, especially when tannins are in high concentration in the sample, and these adducts confirm the molecular weight of analyte (Akande *et al.*, 2022).

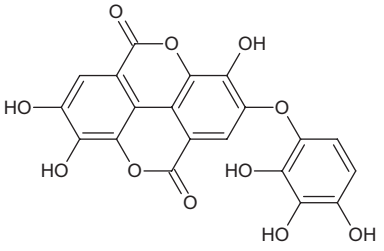
Compound 11, detected as a main compound only in acid hydrolyzed extracts, has not been identified so far. Several attempts were made to identify the chemical nature of this molecule (HPLC-DAD and solid phase extraction [SPE] purification, followed by HPLC-DAD-MS, ESI-ionic trap analysis, matrix-assisted laser desorption ionization (MALDI) analysis, and proton nuclear magnetic resonance [^{1}H-NMR] analysis), described in detail in Supplementary Material. The molecule did not respond to electrospray ionization by applying different fragmentation voltages in negative or positive mode. Furthermore, data from the (^{1}H-NMR) spectroscopy experiments showed the absence of aromatic protons in its molecular structure (see Section S1.4 in Supplementary Material).

Compounds 12 and 13, detected in small amounts in decoction but in higher quantities in the extracts with bicarbonate, were classified as reaction products after rearrangements of intramolecular bonds of punicalagin. They were identified as punicalagin isobars with a molecular fragment at 1,083 m/z and a double charged ion at 541 m/z. Compounds 15 and 23 were referred to as α- and β-form of punicalagin, according to their spectral data and the retention time of standards.

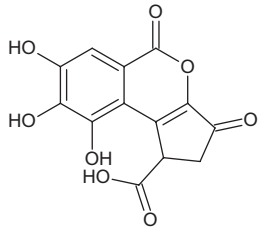
Compound 16, detected in all analyzed extracts, was identified as valoneic acid dilactone in agreement with molecular ion at 469 m/z and the ion derived from decarboxylation at 425 m/z. The same fragmentation pattern was observed for compound 40, identified as an isomer of valoneic acid dilactone, probably sanguisorbic acid dilactone (García-Villalba *et al.*, 2015).

Compound 18, showing the main fragment at 483 m/z, was recognized as digalloylhexoside. This compound was detected in the decoction and in bicarbonate-hydrolyzed samples, but not in acid-hydrolyzed extracts.

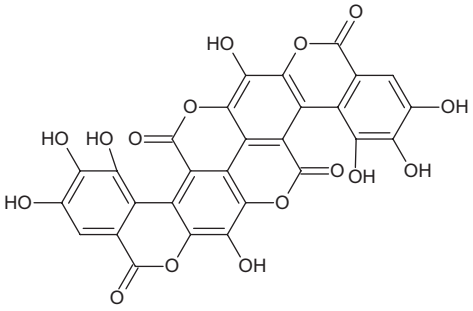
Compounds 19 and 22, with an ion at 1,039 m/z and the corresponding double charged ion at 519 m/z, were identified as isomeric forms derived from the decarboxylation



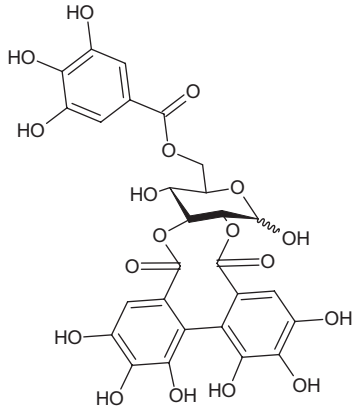
Decarboxylated valoneic acid dilactone



Brevifolin carboxylic acid



Gallagic acid dilactone



Galloyl-HHDP-hexoside

Figure 7. Chemical structure of some phenolic compounds detected in extracts by bicarbonate hydrolysis.

of punicalagin; the molecules were found for the first time only in pomegranate peel extracts from bicarbonate.

Compounds 20 and 33, both with molecular ions at 785 m/z, were identified as isomeric forms of digalloyl-HHDP-hexoside, and together with compound 30 (935 m/z) identified as galloyl-bis-HHDP-hexoside, and were detected in decoction but not in hydrolysates.

Compound 21 was recognized as digalloyl-HHDP-gluconic acid according to the fragment at 801 m/z, attributable to molecular ion. This compound was identified in decoction and in bicarbonate-hydrolyzed samples but not in AH extracts.

Compound 24 was tentatively identified as brevifolin carboxylic acid showing molecular ion at 291 m/z and the ion derived from decarboxylation at 247 m/z; the structure is reported in Figure 7. This EA derivative previously reported in Tanaka *et al.* (1990) is formed starting from DHHDP group.

Compound 26, with a molecular ion at 799 m/z, was tentatively identified as lagerstannin A, previously found in pomegranate juice (Sentandreu *et al.*, 2013).

Compounds 27 and 29 were detected only in acidic extracts and identified as pedunculagin isobars due to a molecular ion at 783 m/z and UV-VIS spectra, very similar to those obtained for pedunculagins. Compounds 32 and 34 were detected in all extracts and identified as α- and β-pedunculagin. It was hypothesized that compounds 27 and 29 are formed after a rearrangement of ester bonds of pedunculagin structure, as observed for punicalins and punicalagins.

Compound 28 was tentatively identified as gallocatechin pentoside, showing a corresponding UV spectrum and a molecular ion with MW of 437 m/z. Presence of this phenolic derivative at low concentration in pomegranate peels was first reported by Plumb *et al.* (2002).

Compounds 36 and 38 were identified as α- and β-anomeric forms of the same ellagitannins digalloyl-gallagyl-hexosides, with a molecular ion at 1,085 m/z and the corresponding double charged ion $[M-2H]^{2-}$at 542 m/z; their UV-VIS spectra were coherent with such a hypothesis.

Compound 39, with molecular ion at 275 m/z, was found in the bicarbonate hydrolysates but not in the decoction and neither in AH extracts; it might be derived from the decarboxylation of EA (Hemingway and Hills, 1971).

Compounds 41, 42, and 46 were identified as isomeric forms of EA monohexoside, with a molecular fragment at 463 m/z; the presence of ion at 301 m/z was in agreement with this hypothesis, corresponding to the loss of sugar moiety. Furthermore, since compound 41 was in higher concentration than other two isobars, it was also possible to detect the dimeric $[2M-H]^-$ ion at 927 m/z, useful for confirming its MW.

Compound 43 was hypothesized to be a derivative of granatin B, formed after the hydrolysis of one of the lactone rings, while compound 44 with a molecular ion at 951 m/z was identified as galloyl-HHDP-DHHDP-hexose or granatin B. This compound was found in decoction and bicarbonate hydrolysates, and in small amounts only in the extracts post-AH after 1 h of reaction time.

Compound 45 was identified as gallagic acid dilactone (molecular ion at 601 m/z), whose structure is reported in Figure 7. Because it was found in bicarbonate hydrolysates and not in decoction, presumably it originated after the hydrolysis of GA glycosidic esters. The compound was also found after acid extraction, resulting as the most abundant compound in solid residue along with EA.

Compound 47 was identified as EA pentoside showing a molecular ion at 433 m/z and ion at 301 m/z derived by the loss of pentoside. Compound 48 was identified as EA by comparison with pure standard. As discussed in Section 3.3, its amount in bicarbonate extracts was significantly higher than in decoction and increased with increase in $NaHCO_3$ concentration. In acid hydrolysates, it was found only in low amounts due to its poor solubility in acidic water, but it was the most abundant component in solid pellet produced after the hydrolysis of peel.

Compound 50, with molecular ion at 425 m/z, was identified as a decarboxylated derivative of valoneic acid dilactone (Figure 7); also as seen for compounds 19, 22, and 39, decarboxylation derivatives are only detected in bicarbonate extracts, confirming that the tannin structure tends to lose carboxyl moiety in the presence of heat and bicarbonate (Hemingway and Hills, 1971).

Conclusions

In this study, bicarbonate-assisted extraction was proposed to give added value to a by-product of pomegranate juice extraction, such as pomegranate peel, naturally rich in ellagitannins.

The composition of the extracts from pomegranate peel obtained after mild alkaline hydrolyses with bicarbonate showed the presence of numerous minor phenols, confirming the presence of glycosidic derivatives as well as

several molecules produced by decarboxylation and rearrangement of native structures.

A high number of phenolic compounds was found in the extracts from mild hydrolyses with $NaHCO_3$, with release of punicalins from punicalagins (partially rearranged, forming new isobaric derivatives), and a higher concentration of EA. After hydrolysis with acid, the concentration of more lipophilic phenols, such as EA and GA, was reduced because of their strong dependence on low solubility in aqueous acid medium. Furthermore, due to a strong acidic environment and high temperature, a lower number of phenols was recovered in the final extracts produced according to a previous procedure, here applied for comparative purposes.

Finally, limited amounts of bound phenols in the peel were found by applying a mild alkaline hydrolysis, confirming that free phenols are largely prevalent in pomegranate. Compared to a simple decoction, the one-step extraction with $NaHCO_3$ proposed in this study allowed obtaining extracts enriched in EA, in phenols with a lower MW than those of native tannins, and in total phenols.

The results of this study paved the way for future investigation of biological activities of these extracts obtainable with simple procedures and enriched in lower MW polyphenols, with potentially greater bioavailability than the native tannins of pomegranate peel, and therefore suitable for the food supplement market.

Acknowledgements

The authors thanked the agricultural companies Azienda Agricola Onori Maria Rosaria and Rio del Sol soc. Agricola for providing peel samples. This research was supported by the Italian Ministry of Education, University and Research (MIUR) and funded by European Union – Next Generation EU (project PRIN2022 MUR, No. 2022X3WZAF).

Author's Contributions

Silvia D'Agostino and Tommaso Ugolini: data curation, formal analyses, investigation, visualization, writing original manuscript, and methodology. Deborah Freschini: data curation and formal analyses. Mohamad Khatib: methodology and formal analyses. Lorenzo Cecchi: investigation, conceptualization, and writing – review and editing. Bruno Zanoni: resources and supervision. Beatrice Zonfrillo: investigation and methodology. Marzia Innocenti: resources and investigation. Maria Bellumori: data curation and methodology. Nadia Mulinacci: conceptualization, investigation, project administration, resources, writing original manuscript, and writing – review and editing. All authors reviewed and approved the final version of the manuscript for publication.

Conflicts of Interest

The authors declared no conflict of interest.

Funding

None.

References

Akande, T., Khatib, M., Ola Salawu, S., Afolabi Akindahunsi, A., Di Cesare Mannelli, L., Ghelardini, C., Balli, D., Cecchi, L. and Mulinacci, N. 2022. 1H NMR and HPLC-DAD-MS for the characterization of ellagitannins and triterpenoids of less investigated Anogeissus leiocarpus DC (Combretaceae) stem bark. Food Chem. 375:131813. https://doi.org/10.1016/j.foodchem.2021.131813

Akuru, E.A., Chukwuma, C.I., Oyeagu, C.E., Erukainure, O.L., Mashile, B., Setlhodi, R., Mashele, S.S., Makhafola, T.J., Unuofin, J.O., Abifarin, T.O. and Mpendulo, T.C. 2022. Nutritional and phytochemical profile of pomegranate ("Wonderful variety") peel and its effects on hepatic oxidative stress and metabolic alterations. J Food Biochem. 46(4):e13913. https://doi.org/10.1111/jfbc.13913

Althunibat, O.Y., Al-Mustafa, A.H., Tarawneh, K., Khleifat, K.M., Ridzwan, B.H. and Qaralleh, H.N. 2010. Protective role of *Punica granatum* L. peel extract against oxidative damage in experimental diabetic rats. Proc Biochem. 45(4):581–585. https://doi.org/10.1016/j.procbio.2009.12.004

Amri, Z., Ghorbel, A., Turki, M., Akrout, F.M., Ayadi, F., Elfeki, A. and Hammami, M. 2017. Effect of pomegranate extracts on brain antioxidant markers and cholinesterase activity in high fat-high fructose diet induced obesity in rat model. BMC Comp Altern Med. 17(1):339. https://doi.org/10.1186/s12906-017-1842-9

Bala, I., Bhardwaj, V., Hariharan, S. and Kumar, M.N.V.R. 2006. Analytical methods for assay of ellagic acid and its solubility studies. J Pharm Biomed Anal. 40(1):206–210. https://doi.org/10.1016/j.jpba.2005.07.006

Balli, D., Cecchi, L., Khatib, M., Bellumori, M., Cairone, F., Carradori, S., Zengin, G., Cesa, S., Innocenti, M. and Mulinacci, N. 2020. Characterization of arils juice and peel decoction of fifteen varieties of *Punica granatum* L.: a focus on anthocyanins, ellagitannins and polysaccharides. Antioxidants. 9(3):238. https://doi.org/10.3390/antiox9030238

Basu, A. and Penugonda, K. 2009. Pomegranate juice: a heart-healthy fruit juice. Nutr Rev. 67(1):49–56. https://doi.org/10.1111/j.1753-4887.2008.00133.x

Cecchi, L., Khatib, M., Bellumori, M., Civa, V., Domizio, P., Innocenti, M., Balli, D. and Mulinacci, N. 2023. Industrial drying

for agrifood by-products re-use: cases studies on pomegranate peel (*Punica granatum* L.) and stoned olive pomace (pâtè, Olea europaea L.). Food Chem. 403:134338. https://doi.org/10.1016/j.foodchem.2022.134338

Dadáková, K., Heinrichová, T., Lochman, J. and Kašparovský, T. 2020. Production of defense phenolics in tomato leaves of different age. Molecules. 25(21):4952. https://doi.org/10.3390/molecules25214952

Dadwal, V., Bhatt, S., Sonkhla, K., Joshi, R. and Gupta, M. 2017. Quantification of free and bound phenolics in biowaste pomegranate peel and formulation of punicalagin rich rice. Int J Food Nutr Sci. 4(2):98–104. https://doi.org/10.15436/2377-0619.17.1321

Dey, D., Ray, R. and Hazra, B. 2015. Antimicrobial activity of pomegranate fruit constituents against drug-resistant Mycobacterium tuberculosis and β-lactamase producing Klebsiella pneumoniae. Pharm Biol. 53(10):1474–1480. https://doi.org/10.3109/13880209.2014.986687

Espín, J.C., Larrosa, M., García-Conesa, M.T. and Tomás-Barberán, F. 2013. Biological significance of urolithins, the gut microbial ellagic acid-derived metabolites: the evidence so far. Evid Based Comp Altern Med. 2013:1–15. https://doi.org/10.1155/2013/270418

Fahmy, H.A. and Farag, M.A. 2022. Ongoing and potential novel trends of pomegranate fruit peel; a comprehensive review of its health benefits and future perspectives as nutraceutical. J Food Biochem. 46(1):e14024. https://doi.org/10.1111/jfbc.14024

Fischer, U.A., Carle, R. and Kammerer, D.R. 2011. Identification and quantification of phenolic compounds from pomegranate (*Punica granatum* L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD–ESI/MSn. Food Chem. 127(2): 807–821. https://doi.org/10.1016/j.foodchem.2010.12.156

García-Villalba, R., Espín, J.C., Aaby, K., Alasalvar, C., Heinonen, M., Jacobs, G., Voorspoels, S., Koivumäki, T., Kroon, P.A., Pelvan, E., Saha, S. and Tomás-Barberán, F.A. 2015. Validated method for the characterization and quantification of extractable and nonextractable ellagitannins after acid hydrolysis in pomegranate fruits, juices, and extracts. J Agric Food Chem. 63(29): 6555–6566. https://doi.org/10.1021/acs.jafc.5b02062

Gu, D., Yang, Y., Bakri, M., Chen, Q., Xin, X. and Aisa, H.A. 2013. A LC/QTOF-MS/MS application to investigate chemical compositions in a fraction with protein tyrosine phosphatase 1B inhibitory activity from *Rosa Rugosa* flowers: chemical compositions in an active fraction from *Rosa Rugosa* flowers. Phytochem Anal. 24(6):661–670. https://doi.org/10.1002/pca.2451

Haghighian, M.K., Rafraf, M., Hemmati, S., Haghravan, S. and Asghari-Jafarabadi, M. 2021. Effects of pomegranate (*Punica granatum* L.) peel extract supplementation on serum lipid profile and oxidative stress in obese women with knee osteoarthritis: a double blind, randomized, placebo controlled study. Adv Integ Med. 8(2):107–113. https://doi.org/10.1016/j.aimed.2020.05.001

Hemingway, R.W. and Hills, W.E. 1971. Behaviour of ellagitannins, gallic acid and ellagic acid under alkaline conditions. J Tech Assoc Pulp Paper Ind. 54(6):933–936.

Hernández-Corroto, E., Marina, M.L. and García, M.C. 2019. Extraction and identification by high resolution mass spectrometry of bioactive substances in different extracts obtained from pomegranate peel. J Chrom A. 1594:82–92. https://doi.org/10.1016/j.chroma.2019.02.018

Khatib, M., Campo, M., Bellumori, M., Cecchi, L., Vignolini, P., Innocenti, M. and Mulinacci, N. 2023. Tannins from different parts of the chestnut trunk (Castanea Sativa Mill.): a green and effective extraction method and their profiling by high-performance liquid chromatography-diode array detector-mass spectrometry. ACS Food Sci Tech. 3(11):1903–1912. https://doi.org/10.1021/acsfoodscitech.3c00272

Khatib, M., Innocenti, M., Giuliani, C., Al- Tamimi, A., Romani, A. and Mulinacci N. 2017. Mesocarp and exocarp of laffan and wonderful pomegranate varieties: by-products as a source of Ellagitannins. Int J Food Nutr Sci. 4(1):60–66. https://doi.org/10.15436/2377-0619.17.1465

Li, Y., Guo, C., Yang, J., Wei, J., Xu, J. and Cheng, S. 2006. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. Food Chem. 96(2): 254–260. https://doi.org/10.1016/j.foodchem.2005.02.033

Li, Y., Ye, T., Yang, F., Hu, M., Liang, L., He, H., Li, Z., Zeng, A., Li, Y., Yao, Y., Xie, Y., An, Z. and Li, S. 2016. *Punica granatum* (pomegranate) peel extract exerts potent antitumor and anti-metastasis activity in thyroid cancer. RSC Adv. 6(87):84523–84535. https://doi.org/10.1039/C6RA13167K

Liu, B., Lei, W., Hu, L. and Zhao, J. 2013. Mild alkaline hydrolysis is an efficient and low-cost method for improving the free phenolic content and health benefit of pomegranate peel extract. J Food Process Preserv. 37:694–700.

Liu, R., Li, J., Cheng, Y., Huo, T., Xue, J., Liu, Y., Liu, J. and Chen, X. 2015. Effects of ellagic acid-rich extract of pomegranates peel on regulation of cholesterol metabolism and its molecular mechanism in hamsters. Food Funct. 6(3):780–787. https://doi.org/10.1039/C4FO00759J

Long, J., Guo, Y., Yang, J., Henning, S.N., Lee, R., Rasmussen, A., Zhang, L., Lu, Q., Heber, D. and Li, Z. 2019. Bioavailability and bioactivity of free ellagic acid compared to pomegranate juice. Food Funct. 10:6582–6588. https://doi.org/10.1039/C9FO01683J

Lv, O., Wang, L., Li, J., Ma, Q. and Zhao, W. 2016. Effects of pomegranate peel polyphenols on lipid accumulation and cholesterol metabolic transformation in L-02 human hepatic cells via the PPARγ-ABCA1/CYP7A1 pathway. Food Funct. 7(12):4976–4983. https://doi.org/10.1039/C6FO01261B

Man, G., Xu, L., Wang, Y., Liao, X. and Xu, Z. 2022. Profiling phenolic composition in pomegranate peel from nine selected cultivars using UHPLC-QTOF-MS and UPLC-QQQ-MS. Front Nutr. 8:807447. https://doi.org/10.3389/fnut.2021.807447

Mastrogiovanni, F., Mukhopadhya, A., Lacetera, N., Ryan, M., Romani, A., Bernini, R. and Sweeney, T. 2019. Anti-inflammatory effects of pomegranate peel extracts on in vitro human intestinal CaCo-2 cells and ex vivo porcine colonic tissue explants. Nutrients. 11(3):548. https://doi.org/10.3390/nu11030548

Mo, F., Lv, B., An, T., Miao, J., Liu, J., Zhang, J., Zhang, Z., Ma, M., Yang, X., Zhao, D., Zhang, D., Gao, S. and Jiang, G. 2019. Protective mechanism of punicalagin against endoplasmic reticulum stress in the liver of mice with type 2 diabetes mellitus. J Funct Foods. 56:57–64. https://doi.org/10.1016/j.jff.2019.03.006

Mourtzinos, I. and Goula, A. 2019. Polyphenols in agricultural byproducts and food waste. In: Polyphenols in Plants. Academic Press, Elsevier, Amsterdam, the Netherlands, pp. 23–44. https://doi.org/10.1016/B978-0-12-813768-0.00002-5

Plumb, G.W., De Pascual-Teresa, S., Santos-Buelga, C., Rivas-Gonzalo, J.C., and Williamson, G. 2002. Antioxidant properties of gallocatechin and prodelphinidins from pomegranate peel. Redox Rep. 7(1):41–46. https://doi.org/10.1179/135100002125000172

Sentandreu, E., Cerdán-Calero, M. and Sendra, J.M. 2013. Phenolic profile characterization of pomegranate (*Punica granatum*) juice by high-performance liquid chromatography with diode array detection coupled to an electrospray ion trap mass analyzer. J Food Comp Anal. 30(1):32–40. https://doi.org/10.1016/j.jfca.2013.01.003

Shahidi, F. and Peng, H. 2018. Bioaccessibility and bioavailability of phenolic compounds. J Food Bioact. 4(7):11–68. https://doi.org/10.31665/JFB.2018.4162

Sun, J., Chu, Y.-F., Wu, X. and Liu, R.H. 2002. Antioxidant and antiproliferative activities of common fruits. J Agric Food Chem. 50(25):7449–7454. https://doi.org/10.1021/jf0207530

Sun, S., Huang, S., Shi, Y., Shao, Y., Qiu, J., Sedjoah, R.-C. A.-A., Yan, Z., Ding, L., Zou, D. and Xin, Z. 2021. Extraction, isolation, characterization and antimicrobial activities of non-extractable polyphenols from pomegranate peel. Food Chem. 351:129232. https://doi.org/10.1016/j.foodchem.2021.129232

Tanaka, T., Nonaka, G. and Nishioka, I. 1990. Tannins and related compounds. C. Reaction of dehydrohexahydroxydiphenic acid esters with bases, and its application to the structure determination of pomegranate tannins, granatins A and B. Chem Pharm Bullettin. 38:2424-2428, https://doi.org/10.1248/cpb.38.2424

Tuominen, A. and Sundman, T. 2013. Stability and oxidation products of hydrolysable tannins in basic conditions detected by HPLC/DAD-ESI/QTOF/MS: stability of hydrolysable tannins in basic conditions. Phytochem Analysis. 24(5):424–435. https://doi.org/10.1002/pca.2456

Venusova, E., Kolesarova, A., Horky, P. and Slama, P. 2021. Physiological and immune functions of punicalagin. Nutrients. 13:2150. https://doi.org/10.3390/nu13072150

Verotta, L., Panzella, L., Antenucci, S., Calvenzani, V., Tomay, F., Petroni, K., Caneva, E. and Napolitano, A. 2018. Fermented pomegranate wastes as sustainable source of ellagic acid: antioxidant properties, anti-inflammatory action, and controlled release under simulated digestion conditions. Food Chem. 246:129–136. https://doi.org/10.1016/j.foodchem.2017.10.131

Xiang, Q., Li, M., Wen, J., Ren, F., Yang, Z., Jiang, X. and Chen, Y. 2022. The bioactivity and applications of pomegranate peel extract: a review. J Food Biochem. 46(7):e14105. https://doi.org/10.1111/jfbc.14105

Zhai, X., Zhu, C., Zhang, Y., Sun, J., Alim, A. and Yang, X. 2018. Chemical characteristics, antioxidant capacities and hepatoprotection of polysaccharides from pomegranate peel. Carbohydr Polym. 202:461–469. https://doi.org/10.1016/j.carbpol.2018.09.013

Zhang, B., Zhang, Y., Li, H., Deng, Z. and Tsao, R. 2020. A review on insoluble-bound phenolics in plant-based food matrix and their contribution to human health with future perspectives. Trends Food Sci Technol. 105:347–362. https://doi.org/10.1016/j.tifs.2020.09.029

Supplementary

Chemical study of Unknown Polar Compound (UPC)

Detection and isolation of UPC through acid hydrolysis (AH)
Unknown polar compounds increased in concentration when hydrolysis time was prolonged, with a maximum of 8 h of hydrolysis. Aiming to identify its chemical structure, the extract after 8 h was used as a source of UPC. As a first step of purification, some interfering compounds were removed by using SPE, in particular a VERSAPAK 40×75 C18 cartridge. A bulk solution of W20M AH 8-h sample (about 500 mL) was prepared, as described in Section 2.3 (in Main Text). The cartridge was wetted with methanol (30 mL) and equilibrated with acid water (200 mL), and the sample was deposited (200 mL). The non-absorbed fraction of the sample was immediately collected and analyzed (sample name UPC-DEP). The first wash of the sample was done with water (approximately 50 mL) and the eluted solution was analyzed (sample name UPC-H_2O). For successive elution steps, 50 mL of mixture with increased concentration of methanol was used: (i) MeOH–H_2O, 30:70 (v/v); (ii) MeOH–H_2O, 70:30 (v/v); and (iii) MeOH. All eluates were analyzed by HPLC-DAD, and fraction with the highest content of UPC was that from MeOH–H_2O, 30:70 (v/v). Figure S1 compares the chromatographic profiles of different eluted fractions from SPE.

As shown in Figure S1, the fraction washed with water was the most concentrated one in UPC, reaching a peak height of above 1750 mAu, but in this solution, the

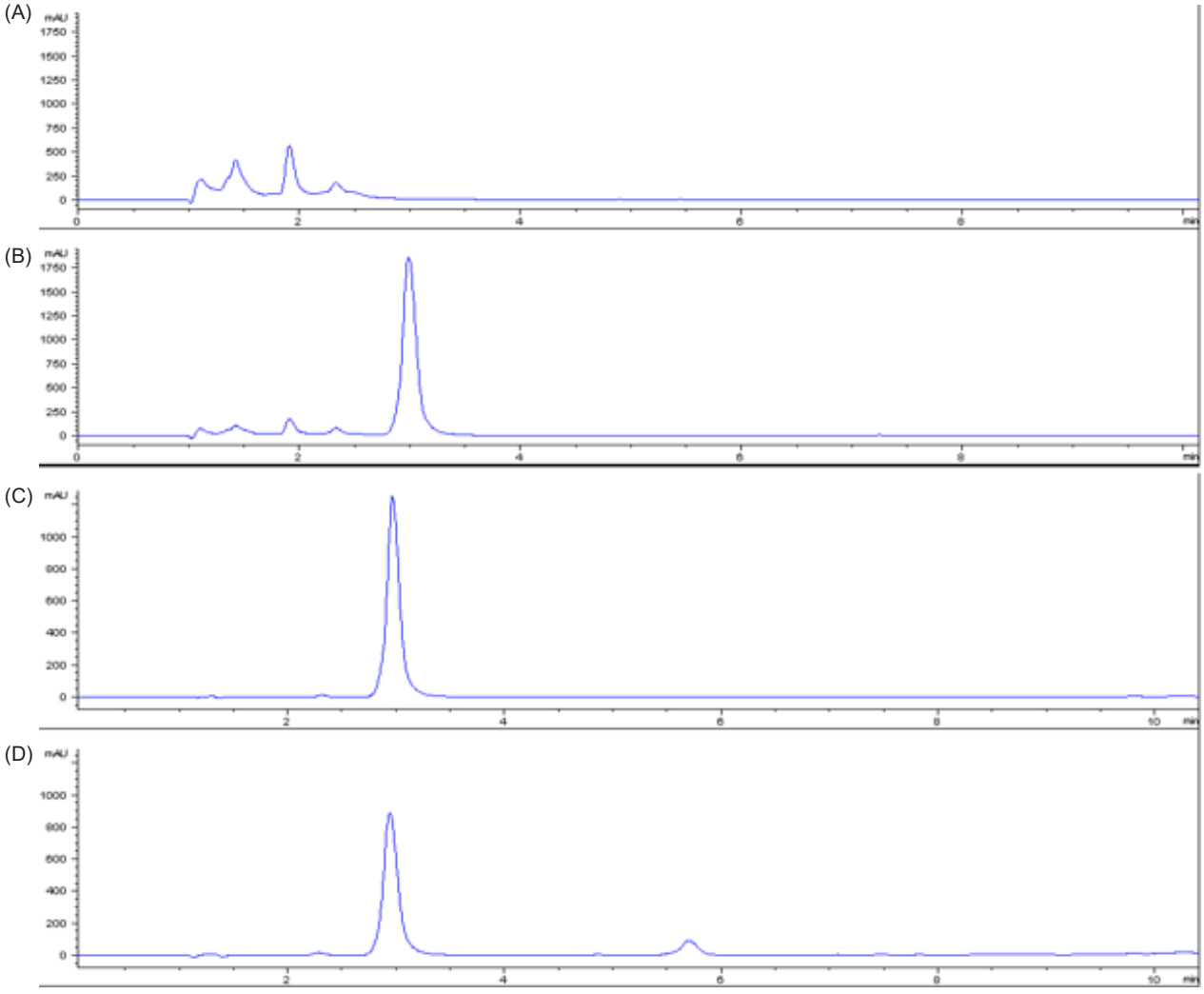


Figure S1. (A) Profile of the whole W20M AH 8-h sample; (B) profile of the sample collected by SPE; (C) first elution with ultrapure water; (D) second elution with MeOH–H_2O, 30:70 (v/v); and (E) third elution with MeOH–H_2O, 70:30 (v/v). All chromatograms are set at 280 nm.

compound of interest was not sufficiently purified. On the other hand, the MeOH–H_2O, 30:70 (v/v) fraction contained UPC compound in lower concentration, but observing the profiles at different wavelengths (data not shown), it resulted as completely purified. This new purified sample, named UPC, showed UV-Vis spectrum as depicted in Figure S2 and was used for further analysis.

Flow injection analysis for mass determination through HPLC-DAD-MS

Initial analysis by HPLC-DAD-MS with a fixed fragmentor power (150 V) was conducted to determine the molecular weight of UPC, but no ionization was observed in any of the extracts in which the compound was presented. To optimize fragmentor's ionization efficiency, a flow injection analysis coupled to mass spectrometry (FIA-MS) was performed on UPC. In this type of analysis, the sample is repeatedly injected and analyzed, with settings differing only for fragmentor voltage. In this way, a wide spectrum of voltages was tested in both positive and negative ionization modes. It was possible to identify fragmentor's settings that allowed maximum ion current for the injected sample, and therefore the best conditions for the ionization of sample were selected. In Figure S3, the FIA-MS chromatograms (or FIAgrams) are shown.

As shown in Figure S3, in negative ionization mode, maximum ionization (more intense) was reached with fragmentor at 110 V, and for positive ionization mode, maximum ionization was reached with fragmentor at 170 V. Nevertheless, analyses performed by applying these fragmentors did not produce enough ionization to extrapolate the mass spectrum of UPC (Figure S4).

Mass analysis of UPC by MALDI

A further attempt to ionize UPC was carried out with matrix-assisted laser desorption ionization (MALDI) to verify the possible polymeric nature of UPC. UPC, 1 mg/mL solution, was deposited on a MALDI plate with a α-cyano-4-hydroxycinnamic acid (HCCA) matrix, and the experiment was carried out in negative ionization mode. Figure S5 shows only some low-intensity fragments at a relatively low molecular weight (MW: 621.1, 648.8, 670.7, 861.2, and 898.5 m/z), but these fragments were not useful to identify UPC. Furthermore, the very intensity of the ions was not coherent with the amount of the sample deposited on the plate. Therefore, the observed ions were probably generated from impurities co-present in the sample. The non-ionizable characteristics of UPC confirms our hypothesis of its non-phenolic nature.

^{1}H-NMR spectroscopy for determination of structure

As no information was collected through mass spectrometry, different experiments through nuclear magnetic resonance (NMR) spectroscopy were carried out. The UPC solution purified by SPE, as described previously, was dried with a rotary evaporator; the residual solid was dissolved in 1 mL of water, and freeze-dried. The powder thus obtained (9 mg) was dissolved in 1-mL D_2O for ^{1}H-NMR analysis.

No signals were present at chemical shifts higher than 5 ppm, indicating the absence of aromatic hydrogens. The signal at δ = 1.1 (triplet) and δ = 3.6 (quadruplet) suggested the presence of an ethoxy group, while the singlet at δ = 3.3 indicated a methylene bridge, and two

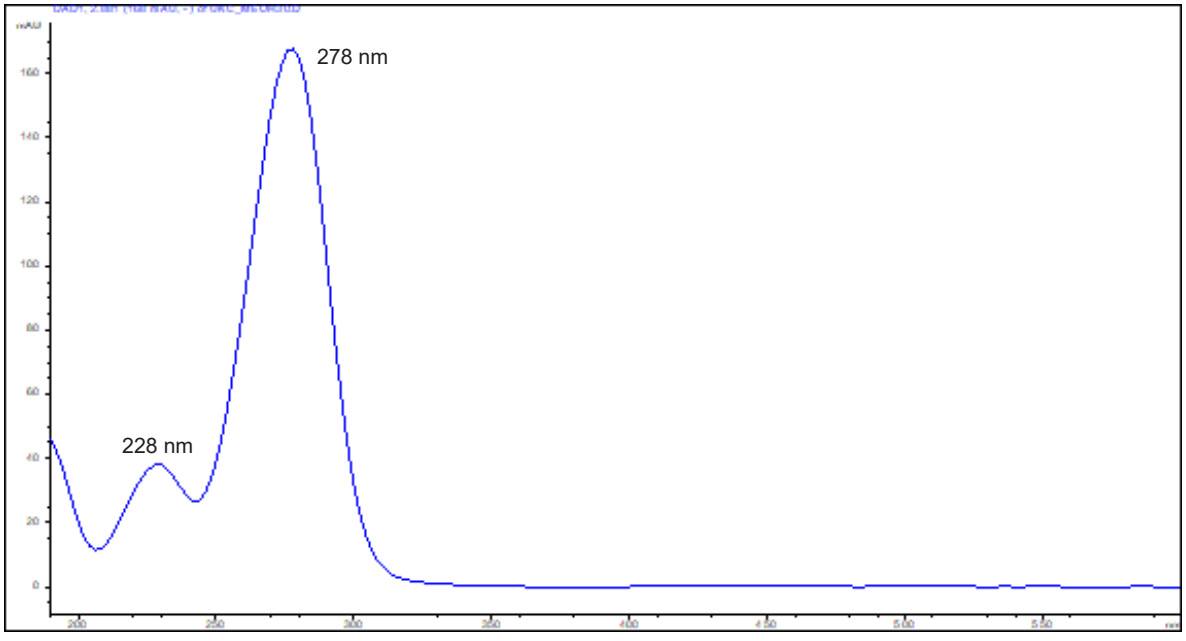


Figure S2. UPC UV/Vis absorbance spectrum.

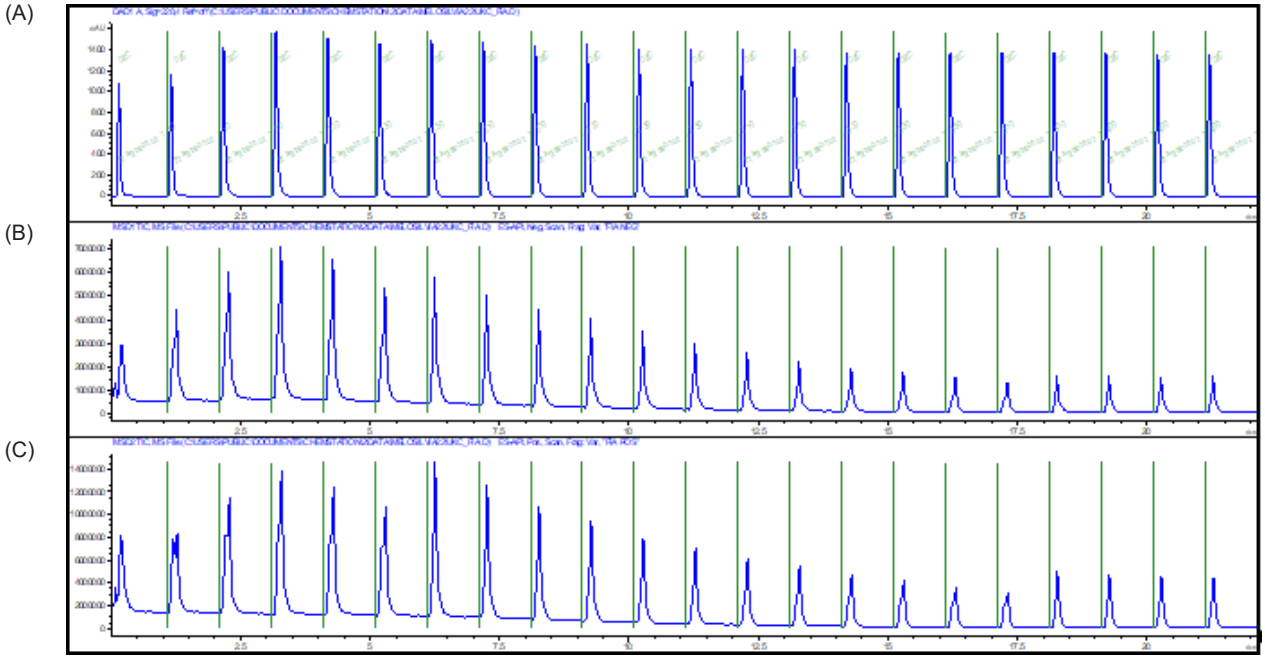


Figure S3. (A) UPC FIAgram (HPLC-DAD) at 280 nm; (B) UPC FIAgram (MS) in negative ionization mode; (C) UPC FIAgram (MS) in positive ionization mode.

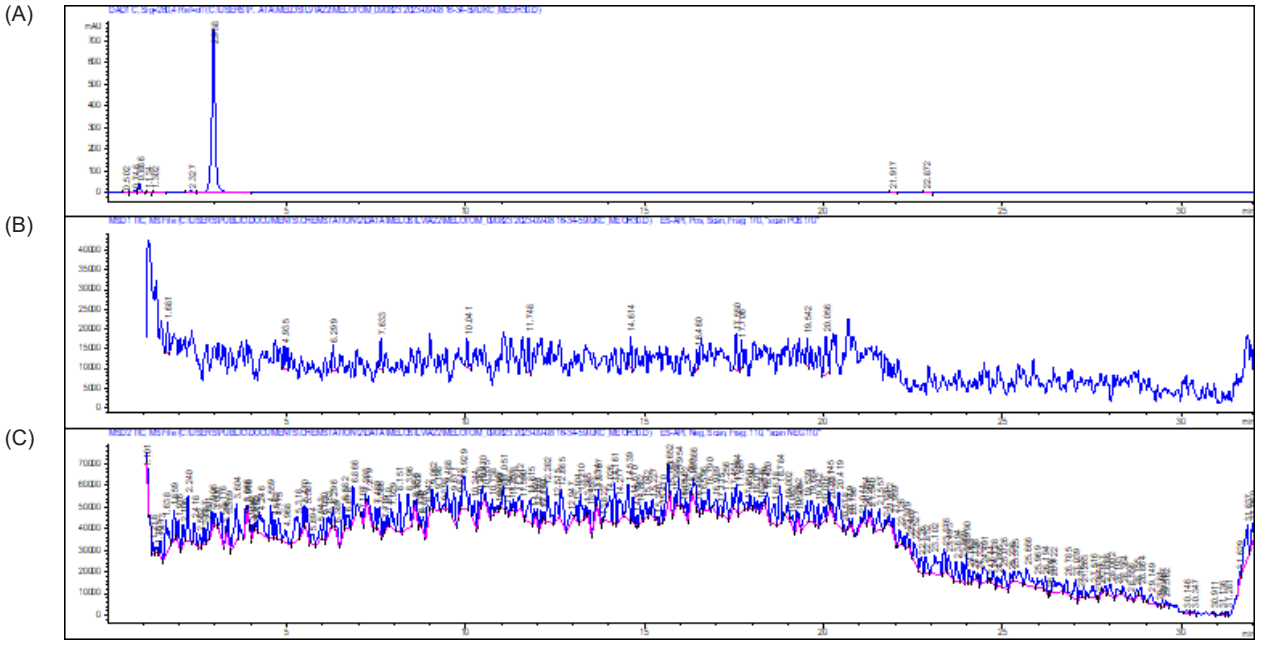


Figure S4. (A) HPLC-DAD chromatogram of UPC at 280 nm; (B) TIC profile in negative ionization mode with fragmentor at 110 V; (C) TIC profile in positive ionization mode with fragment at 170 V. TIC: total ion chromatogram.

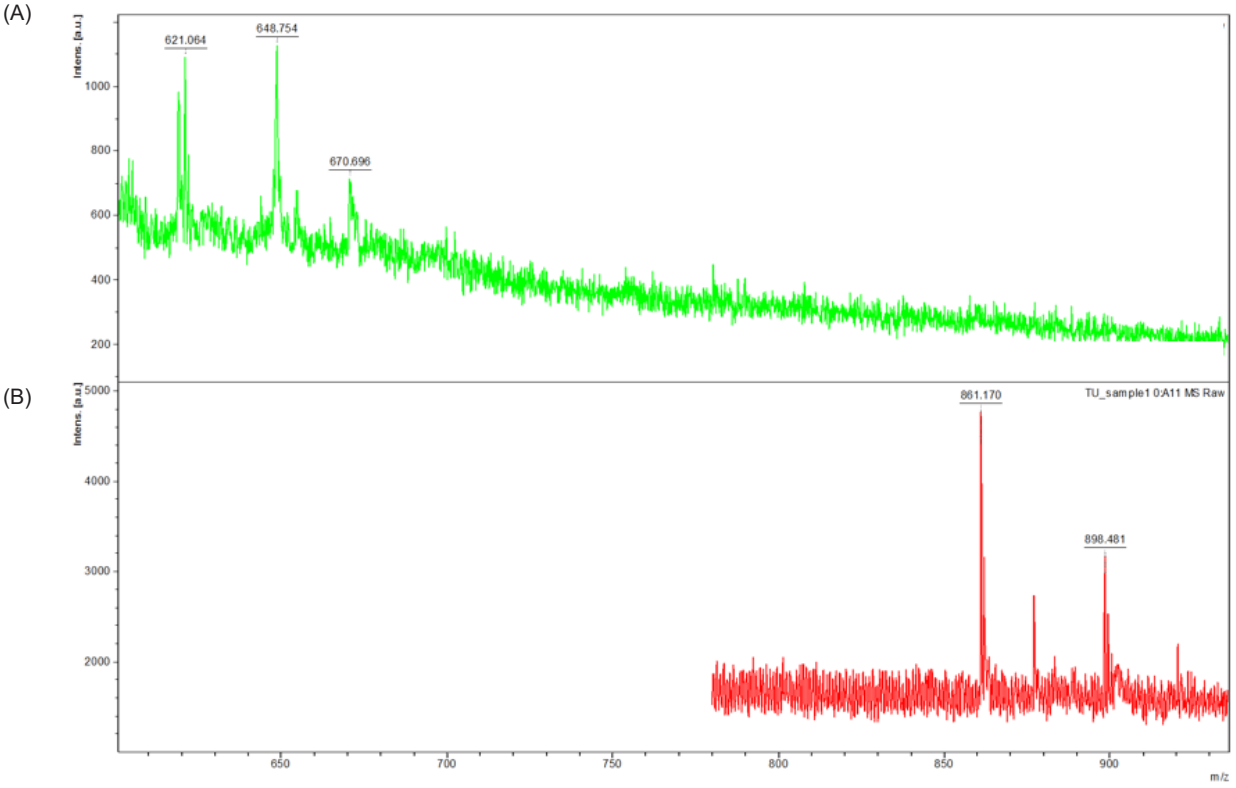


Figure S5. MALDI-MS spectra of UPC in negative ionization mode: (A) in green, the spectrum for low molecular weight fragments; (B) in red, the spectrum for higher molecular weight fragments.

triplets at δ = 2.35 and δ = 2.7, integrating 1 proton each, seem to belong to two correlating CH groups. Signals at δ = 4.6 and δ = 2.15 and satellites at δ = 2 and δ = 2.3 were the solvent's residual signals and did not belong to UPC. This data so far without other information by mass spectrometry did not permit to identify the chemical structure of UPC. Further analysis could be conducted through ^{13}C-NMR and bi-dimensional NMR spectroscopy to collect suitable data to understand the structure of this compound.

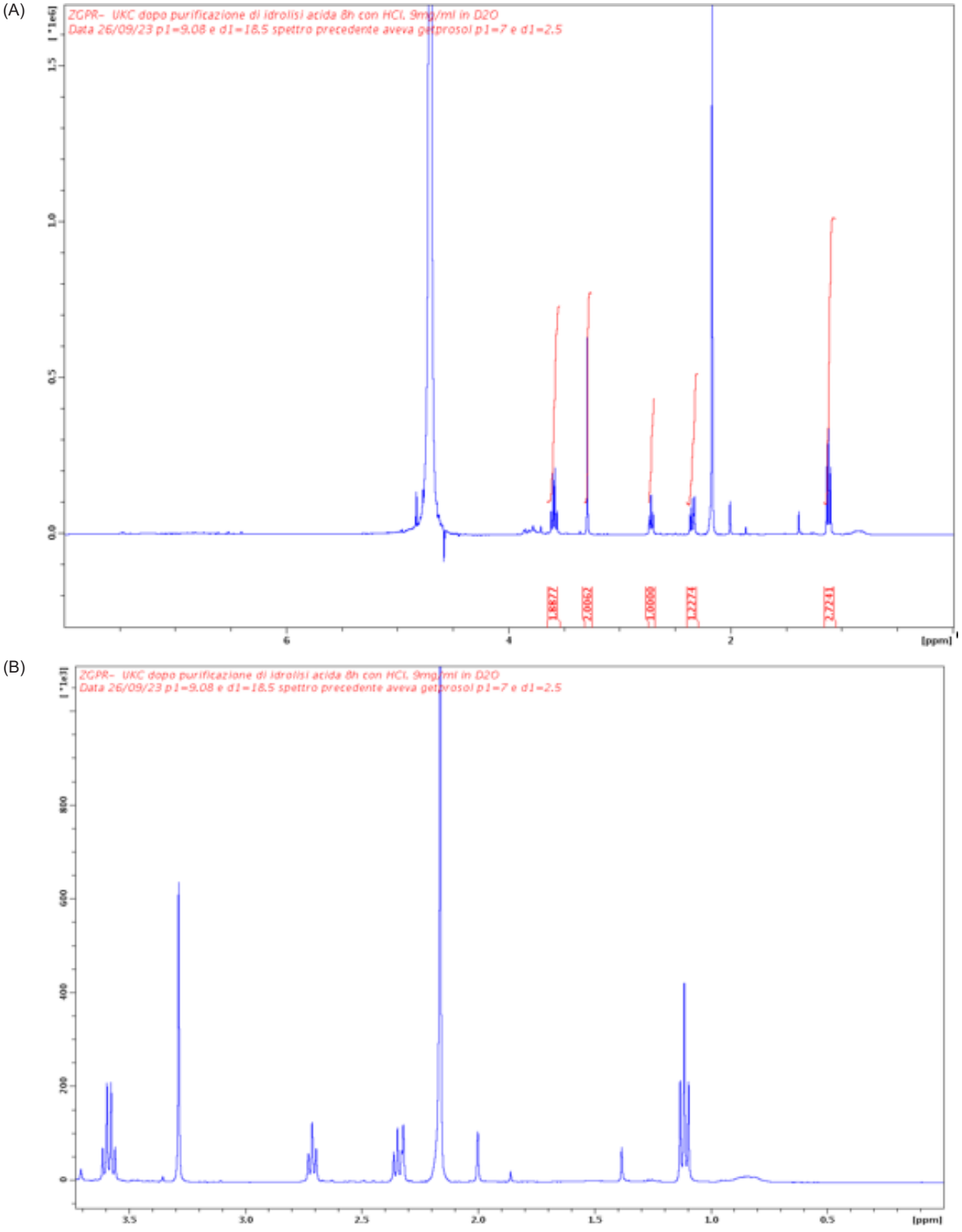


Figure S6. (A) ^{1}H-NMR spectrum of UPC sample in D_2O; (B) zoom from 0 to 3.8 ppm.

Table S1. Concentration of main compounds found in the washing samples of G20M and W20M varieties through AH at different time. Comparison between two different washing mixtures.

Compound	Samples	4 h	4 h	8 h	8 h
		DMSO–MeOH (50:50)	EtOH–H_2O (80:20)	DMSO–MeOH (50:50)	EtOH–H_2O (80:20)
Residue after AH (mg/g DM)					
UPC	G20M	n.d.	3.6 ± 1.3	n.d.	7.6 ± 0.8
	W20M	n.d.	2.9 ± 0.9	n.d.	7.7 ± 1.0
Ellagic acid-hexoside isomer 1	G20M	n.d.	1.0 ± 0.1	n.d.	n.d.
	W20M	n.d.	2.1 ± 0.2	n.d.	n.d.
Valoneic acid dilactone	G20M	9.2 ± 0.4	3.3 ± 0.2	10.3 ± 4.8	5.9 ± 0.2
	W20M	6.0 ± 0.8	3.5 ± 0.2	6.1 ± 0.5	5.3 ± 0.8
Gallagic acid	G20M	9.4 ± 1.1	2.5 ± 0.2	35.2 ± 4.7	6.2 ± 0.5
	W20M	10.5 ± 1.1	3.8 ± 0.2	43.3 ± 1.4	6.8 ± 3.9
Ellagic acid-hexoside isomer 2	G20M	n.d.	1.0 ± 0.1	n.d.	1.4 ± 0.1
	W20M	n.d.	1.5 ± 0.1	n.d.	1.5 ± 0.6
Ellagic acid	G20M	72.5 ± 2.6	22.5 ± 1.3	83.0 ± 11.0	26.8 ± 2.7
	W20M	59.6 ± 2.0	21.9 ± 0.8	69.5 ± 2.8	25.8 ± 3.4
Total content	G20M	91.1 ± 4.0	34.0 ± 0.5	128.4 ± 19.7	48.9 ± 2.1
	W20M	76.2 ± 2.1	35.7 ± 0.8	118.9 ± 3.9	49.6 ± 2.0

Table S2. Data processing of main chemicals detected after (A) acid and (B) alkaline hydrolyses.

(A)	Sample	Time	Sa × Ti
UPC	***	***	N.S.
Ellagic acid hexoside isomer 1	***	***	***
Valoneic acid dilactone	***	***	***
Gallagic acid	**	***	N.S.
Ellagic acid hexoside isomer 2	***	*	N.S.
Ellagic acid	***	N.S.	*
Total compounds	***	***	***
(B)	**Sample**	**Concentration**	**Sa × Co**
Gallic acid	N.S.	***	***
α-punicalin	***	***	***
β-punicalin	***	*	*
Punicalagin isobar	***	***	***
α-punicalagin	**	***	N.S.
β-punicalagin	***	***	N.S.
Ellagic acid hexoside	***	***	N.S.
Ellagic acid	**	***	***
Total compounds	***	**	*

Notes: For each molecule/group of molecules, results from two-factor ANOVA were reported, where the factors were the sample and time for AH, and sample and $NaHCO_3$ concentration for alkaline hydrolysis. Two-way interactions were also reported.
For each parameter, ***significant effect at $p < 0.001$, **significant effect at $p < 0.01$, *significant effect at $p < 0.05$.
N.S.: nonsignificant effect.

Table S3. Phenols recovered in the washing samples of the precipitates of G20M variety sample after AH and washing of solid residue.

Sample	Compound	4 h	8 h
Residue after AH (mg/g DM)			
G20M	UPC	3.55 ± 1.35^{c}	7.61 ± 0.84^{a}
	EA-hesoxide isomer 1	1.02 ± 0.07^{b}	n.d.
	Valoneic acid dilactone	3.30 ± 0.22^{c,d}	5.86 ± 0.20^{a}
	Gallagic acid	2.55 ± 0.21$^{c–e}$	6.19 ± 0.49^{a,b}
	EA-hesoxide isomer 2	1.03 ± 0.01^{b,c}	1.38 ± 0.12^{a,b}
	Ellagic acid	22.5 ± 1.3^{a,b}	26.8 ± 2.7^{a}
	Total content	**34.0 ± 0.5**^{b}	**48.9 ± 2.1**^{a,b}

Notes: n.d.: not detected.

Table S4. Concentration of main phenols found in the: decoction (D) of W20M variety sample, washing solutions (Wash 1 and Wash 2), and hydrolyzed extracts of the residue from decoction (AH and B2) obtained as described in Section 3.4.

Compounds (mg/g DM)	W20M-D	Wash 1	Wash 2	W20M-AH (R)*	W20M-B2 (R)*
Gallic acid	0.6 ± 0.1	0.08 ± 0.01	-	-	-
α-punicalin	4.4 ± 0.2	0.4 ± 0.1	tr	-	0.3 ± 0.1
β-punicalin	4.8 ± 0.3	0.4 ± 0.1	tr	-	0.3 ± 0.1
Punicalagin isobar	11.5 ± 0.2	-	-	-	-
α-punicalagin	49.5 ± 2.4	6.1 ± 1.4	0.8 ± 0.4	-	0.7 ± 0.1
β-punicalagin	86.5 ± 4.5	8.4 ± 2.0	1.2 ± 0.6	-	1.3 ± 0.2
Ellagic acid hexoside	1.1 ± 0.1	0.14 ± 0.04	tr	-	0.7 ± 0.1
Ellagic acid	2.3 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	-	2.8 ± 0.2
Total content	**160.7 ± 7.9**	**16.1 ± 3.8**	**2.4 ± 1.1**		**6.0 ± 0.7**

Notes: Data are a mean value of triplicate and expressed as mg/g DM.
*Hydrolysis of washed residue.
tr: trace amounts.