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Analytical Methods

Identification and quantification of phenolic compounds from pomegranate (*Punica granatum* L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD–ESI/MS^n

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ABSTRACT

Phenolic compounds were extracted from pomegranate (*Punica granatum* L.) peel, mesocarp and arils. Extracts and juices were characterised by HPLC-DAD–ESI/MS^n. In total, 48 compounds were detected, among which 9 anthocyanins, 2 gallotannins, 22 ellagitannins, 2 gallagyl esters, 4 hydroxybenzoic acids, 7 hydroxycinnamic acids and 1 dihydroflavonol were identified based on their UV spectra and fragmentation patterns in collision-induced dissociation experiments. To the best of our knowledge, cyanidin–pentoside–hexoside, valoneic acid bilactone, brevifolin carboxylic acid, vanillic acid 4-glucoside and dihydrokaempferol-hexoside are reported for the first time in pomegranate fruits. Furthermore, punicalagin and pedunculagin I were isolated by preparative HPLC and used for quantification purposes. The ellagitannins were found to be the predominant phenolics in all samples investigated, among them punicalagin ranging from 11 to 20 g per kilogram dry matter of mesocarp and peel as well as 4–565 mg/L in the juices. The isolated compounds, extracts and juices were also assessed by the TEAC, FRAP and Folin–Ciocalteu assays revealing high correlation (R^2 = 0.9995) of the TEAC and FRAP values, but also with total phenolic contents as determined by the Folin–Ciocalteu assay and by HPLC. Selection of raw materials, i.e. co-extraction of arils and peel, and pressure, respectively, markedly affected the profiles and contents of phenolics in the pomegranate juices, underlining the necessity to optimise these parameters for obtaining products with well-defined functional properties.

1. Introduction

Pomegranates are cultivated around the world in subtropical and tropical regions with different microclimatic zones such as in Iran, California, Turkey, Egypt, Italy, India, Chile and Spain. The world pomegranate production amounts to approximately 1,500,000 tons with Iran contributing 47% to the total. The pomegranate export of Iran increased from 14,075 tons in 2003 to 27,439 tons in 2007, thus proving the soaring demand for pomegranates and products derived therefrom, such as juices, jams and wines. The market has steadily grown, which is presumably due to the increasing consumer awareness of the potential health benefits attributed to pomegranates and phytochemicals thereof (FAOSTAT-FAO, 2010).

Plant based diets rich in phytochemicals have been associated with a reduced risk of diseases such as certain types of cancer, inflammation, cardiovascular and neurodegenerative diseases. Therefore, the characterisation and identification of phytochemicals are of utmost importance to substantiate their potential health benefits in human nutrition (Bravo, 1998; Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005). Polyphenols represent the predominant class of phytochemicals of pomegranate fruits mainly consisting of hydrolysable tannins. Phenolic compounds may exert beneficial effects through their free radical scavenging and antioxidant potential. Among these are hydrolysable tannins, which are mainly located in the fruit peel and mesocarp of pomegranates. They are extracted into the juice upon commercial processing of the whole fruits (Gil, Tomás-Barberán, Hess-Pierce, Holcroft, & Kader, 2000). The tannin fraction consists of gallic acid and ellagic acid esters of core polyol molecules. The large number of possible combinations of monomers gives rise to enormous structural diversity. These structures are subdivided into gallotannins (type I-tannins) and ellagitannins (type II-tannins) including the more unique gallagylesters, such as the predominant hydrolysable tannin of pomegranates known as punicalagin (Haslam, 2007; Khanbabaee & van Ree, 2001; Okuda, Yoshida, & Hatano, 2000). Further classes of ellagitannins comprise dehydroellagitannins (type III-tannins) and transformed dehydroellagitannins (type IV-tannins) (Okuda et al., 2000).

Polyphenols are important constituents regarding the organoleptic properties of pomegranate arils and juices as they impart

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the appealing red colour and provide mild astringency that is characteristic of pomegranate flavour. A number of methods for the determination of polyphenolics such as punicalagin, punicalin and other ellagitannins in pomegranates and products derived therefrom have been reported (Gil et al., 2000); however, a detailed and comprehensive characterisation of gallotannins, ellagitannins and of low-molecular phenolics by mass spectrometry has not been reported so far. Although the identification of some specific gallotannins and ellagitannins, such as punicacortein, punigluconin or galloylpunicalin by NMR spectroscopy, has been described, these compounds may only be found in the bark and leaves of pomegranate trees (Tanaka, Nonaka, & Nishika, 1985, 1986a, 1986b). Furthermore, exhaustive quantitative data on the contents of individual phenolic compounds including predominant and minor components in pomegranate fruits and juices are still lacking.

Therefore, the main objective of the present study was to develop an HPLC method for the systematic determination of individual phenolic compounds, especially tannins, in pomegranates. Due to the limited availability of reference substances, and since the identification of structurally related compounds solely based on UV spectra may prove difficult, particular attention should be given to the compatibility of the HPLC eluents with mass spectrometric detection. Standard compounds of punicalagin and pedunculagin I (bis-hexahydroxydiphenoyl-hexoside, bis-HHDP-hexoside) should be isolated by preparative HPLC in order to quantify individual tannins. The methodology should be applicable for the characterisation and quantification of phenolic compounds in pomegranate fruit (peel, mesocarp and arils) and in pomegranate juices made from isolated arils and the entire fruit, respectively.

2. Materials and methods

2.1. Solvents and reagents

All reagents and solvents were of analytical or HPLC grade. TPTZ [2,4,6-tripyridyl-s-triazine], trolox [(+/−)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid], $FeCl_3{*}6H_2O$, ABTS [2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt], ABAP [2,2′-azo-bis-(2-amidino-propane) dihydrochloride] and Folin–Ciocalteu reagent were purchased from Sigma (St. Louis, MO, USA). C_{18} end capped reversed-phase cartridges (Chromabond, 1000 mg) were obtained from Macherey-Nagel (Düren, Germany). The following standards were used for identification and quantification purposes with HPLC–MS and HPLC-DAD, respectively: cyanidin 3-glucoside, delphinidin 3-glucoside, pelargonidin 3-glucoside, cyanidin 3-rutinoside (Polyphenols, Sandnes, Norway); p-coumaric acid, ferulic acid, caffeic acid, protocatechuic acid, chlorogenic acid (Roth, Karlsruhe, Germany); gallic acid (Fluka, Buchs, Switzerland); ellagic acid (Serva, Heidelberg, Germany); dihydroquercetin hydrate (Sigma, St. Louis, MO, USA); and punicalagin and pedunculagin I (isolated by preparative HPLC, see Section 2.3). All other reagents and solvents were purchased from VWR (Darmstadt, Germany). Deionised water was used throughout.

2.2. Sample preparation

Peruvian pomegranate fruits of unknown cultivar obtained from the local market were washed, steamed (5 min) for enzyme inactivation and subsequently pressed with a rack and cloth press (Wahler, Stuttgart, Germany) to obtain two different juices. Juice variant 1 was prepared by applying a pressure of 10 bar, whereas variant 2 was obtained at an elevated pressure of 150 bar. Additionally, the fruits were manually separated into leathery peel (exocarp), fleshy mesocarp and seeds with surrounding arils, and a third juice variant was solely prepared from the isolated arils which are coating the seeds using a Hafico tincture press (Fischer Maschinenfabrik, Neuss, Germany) at 250 bar.

Peels and mesocarp were separately lyophilised and ground with a blender (Waring Products Division, Torrington, CT, USA). The homogenised powder was extracted with aqueous methanol (80%, v/v; 0.1% HCl) after flushing with nitrogen for 30 min at ambient temperature under continuous stirring. After filtration through a filter paper, the extracts were evaporated to dryness *in vacuo* at 30 °C, and the residue was dissolved in 10 mL of acidified water (pH 3.0). Anthocyanins were analysed by direct injection of crude extracts and juices after centrifugation (15 min; 17,661 g; MiniSpin plus, Eppendorf-Netheler-Hinz, Hamburg, Germany), respectively, without further sample purification. Non-anthocyanin phenolics of peel and mesocarp extracts and pomegranate juices were extracted with ethyl acetate prior to fractionation via solid phase extraction (SPE). For this purpose, aliquots of 5 mL of juice and peel and mesocarp extracts were made up to 20 mL, and the pH was adjusted to 1.5 with diluted HCl. The solutions were extracted fourfold with 50 mL ethyl acetate each, and the combined extracts were evaporated to dryness, dissolved in water and applied to the SPE cartridges after pH adjustment to 7.0.

The SPE cartridges were activated with 3 mL of methanol and subsequently rinsed with 10 mL of deionised water (Kammerer, Claus, Carle, Schieber, 2004b). Hydrophilic pomegranate phenolics were subsequently eluted with 10 mL of deionised water and 10 mL of 0.01% HCl (fraction I), whereas fraction II was obtained by elution with 20 mL of ethyl acetate. The eluates were concentrated *in vacuo*, and the residues obtained were dissolved in 2% acetic acid (fraction I) and methanol (fraction II), respectively, membrane-filtered (0.45 μm), and used for LC analysis.

2.2.1. Acid hydrolysis of ellagic acid derivatives

Acid hydrolysis of pomegranate juice (variant 1) was performed according to a modified procedure previously applied for the characterisation of anthocyanins in black carrots (Kammerer, Carle, & Schieber, 2003). After extraction with ethyl acetate, the combined extracts were evaporated to dryness, dissolved in water and mixed with 10 mL of 2 mol/L HCl. The ellagic acid derivatives were hydrolyzed for 30 min at 95 °C under nitrogen atmosphere. After cooling on an ice-bath, the solution was adjusted to pH 3.0 with 20% (w/v) KOH and evaporated *in vacuo*. The residue was dissolved in 5 mL 0.01% HCl and membrane-filtered (0.45 μm) prior to HPLC and LC–MS analysis.

2.3. Preparative isolation of reference compounds

Punicalagin and pedunculagin I were quantified with standard compounds isolated by preparative HPLC. For this purpose, pomegranate peels were lyophilised and ground with a Grindomix GM 200 knife mill (Retsch, Haan, Germany). An aliquot of 34 g of homogenised peel powder was extracted with 1 L of aqueous methanol (80%, v/v; 0.1% HCl) after flushing with nitrogen for 60 min at ambient temperature and under continuous stirring. After filtration through a filter paper, the extract was evaporated to dryness *in vacuo* at 30 °C, and the residue was dissolved in 10 mL of acidified water (pH 3.0). The standards were isolated using a Bischoff HPLC system controlled by McDAcq32 Control software (Version 2.0; Bischoff, Leonberg, Germany) equipped with an LCCaDI 2214 controller, two solvent delivery modules, a 2250 HPLC compact pump and a SPD 10AVVp UV/Vis detector (Shimadzu Corporation, Kyoto, Japan). Separation was performed with a preparative Phenomenex Aqua C18 reversed phase column (250 × 21.1 mm i.d., particle size 5 μm; 125 Å pore size). The mobile phase consisted of 2% (v/v) acetic acid in water (eluent A) and of 0.5% acetic acid in water and methanol (10/90, v/v; eluent

B) using a linear gradient as follows: 0–2% B (13 min), 2–5% B (5 min), 5–10% B (5 min), 10–25% B (21 min), 25–100% B (5 min), 100% B isocratic (5 min), 100–0% B (5 min) at a constant flow rate of 7 mL per min at 20 °C. Total run time was 59 min, with retention times of 29 min for pedunculagin I and 40 min for punicalagin. Aliquots of 500 μL of the pomegranate peel extract were used for each HPLC run. Monitoring was performed at 280 nm.

The collected fractions were evaporated to dryness (T_{max} = 30 °C), weighed, dissolved in a defined volume of methanol and identified by mass spectrometry (peak purity see Section 3.3 and Fig. 6). Isolated compounds were used for quantification purposes (calibration range 5–1000 mg/L).

2.4. HPLC-DAD analysis

Polyphenol analyses were performed using a Merck Hitachi La-Chrom Elite HPLC system (Merck, Darmstadt, Germany) equipped with an L-2200 autosampler, an L-2130 pump, a Jetstream column oven, and an L-2450 diode array detector. The separation was carried out with an analytical Phenomenex (Torrance, CA, USA) C 18 Synergi 4 μm Hydro-RP 80 Å pore size (150 × 3.0 mm) column fitted with a Phenomenex (Torrance, CA, USA) security guard column (4 × 3.0 mm) operated at 30 °C. The diode array detector was set at an acquisition range of 200–600 nm.

2.4.1. System I (anthocyanins)

The mobile phase consisted of 5% (v/v) formic acid in water (eluent A) and of water, formic acid and methanol (10/10/80, v/v/v; eluent B). The flow rate was 0.4 mL/min, and the gradient programme was optimised as follows: 10–14% B (5 min), 14–23% B (11 min), 23–35% B (5 min), 35–40% B (14 min), 40–100% B (3 min), 100% B isocratic (3 min), 100–10% B (3 min), 10% B isocratic (4 min). Total run time was 48 min. The injection volume for all samples was 10 μL. Monitoring was performed at 520 nm.

2.4.2. System II (non-anthocyanin phenolics)

The mobile phase consisted of 2% (v/v) acetic acid in water (eluent A) and of 0.5% acetic acid in water and methanol (10/90, v/v; eluent B). The flow rate was 0.4 mL/min, and the gradient programme was optimised as follows: 0–2% B (13 min), 2–5% B (5 min), 5–10% B (5 min), 10–25% B (20 min), 25–50% B (10 min), 50–100% B (5 min), 100% B isocratic (5 min), 100–0% B (3 min), 0% B isocratic (5 min). Total run time was 71 min. The injection volume for all samples was 15 μL. Simultaneous monitoring was performed at 280 nm and 320 nm.

2.5. LC–MS analysis

LC–MS analyses were carried out using an Agilent HPLC 1100 system (Agilent, Waldbronn, Germany) equipped with ChemStation software, a model G1379A degasser, a model G1312A binary gradient pump, a model G1313A thermoautosampler, a model G1316A column oven, and a model G1315A diode array detection system. The HPLC system was connected in series with a Bruker (Bremen, Germany) model Esquire 3000+ ion trap mass spectrometer fitted with an ESI source. Data acquisition and processing were performed using Esquire Control software. Positive ion (anthocyanins, system I) and negative ion (non-anthocyanin phenolic compounds, system II) mass spectra of the column eluate were recorded in the range of m/z 50–1500 (system I) and m/z 50–2800 (system II) at a scan speed of 13,000 $m/z/s$. Nitrogen was used both as drying gas at flow rates of 10.0 L/min (system I) and 9.0 L/min (system II), respectively, and nebulising gas at pressures of 50.0 psi (system I) and 40.0 psi (system II). The nebuliser temperature was set at 365 °C. Helium was used as collision gas at a pressure of 4×10^{-6} mbar.

2.6. Quantification of individual phenolic compounds by HPLC-DAD

Individual phenolic compounds were quantified using calibration curves of the respective reference compounds. For this purpose, stock solutions (1.000 mg/L) were diluted to concentrations of 0.5–500 mg/L (ellagic acid, protocatechuic acid, gallic acid, chlorogenic acid, caffeic acid, coumaric acid and dihydroquercetin hydrate), 0.5–200 mg/L (ferulic acid), 0.25–10 mg/L (pelargonidin 3-glucoside and cyanidin 3-rutinoside) and 0.25–500 mg/L (cyanidin 3-glucoside and delphinidin 3-glucoside), respectively, and the solutions were analysed as described in Section 2.4. The reference substances punicalagin and pedunculagin I (bis-hexahydroxydiphenoyl-hexoside, bis-HHDP-hexoside) were isolated by preparative HPLC (Section 2.3). When reference compounds were not available, the calibration was based on structurally related substances, using a molecular weight correction factor (Chandra, Rana, & Li, 2001) (gallic acid for compounds B1, B2 and E4; ellagic acid for C3–C5, C11–C14, C16 and C22; bis-HHDP-hexoside for C2, C6–C9, C15 and C17–C21; punicalagin for D1; protocatechuic acid for E3; caffeic acid for F1–F3; coumaric acid for F5; ferulic acid for F6; dihydroquercetin hydrate for G1).

2.7. Quantification of antioxidant activity

To assess the antioxidant potential of bioactive compounds, the application of at least two different assays varying in their mechanisms of antioxidant action has been recommended (Schlesier, Harwat, Böhm, & Bitsch, 2002). The antioxidant capacity of pomegranate juice, peel and mesocarp was determined applying the TEAC (trolox equivalent antioxidant capacity) and FRAP (ferric reducing antioxidative power) assays. While the TEAC test allows the direct determination of the radical scavenging capacity of plant extracts or individual compounds by reducing the ABTS radical, the FRAP assay monitors antioxidants as reducing agents in a redox-linked colorimetric reaction. Trolox was used as a reference in both assays (calibration range 5–60 mg/L for the TEAC-assay and 0.5–30 mg/L for the FRAP-assay, respectively), and the antioxidant capacity was calculated as μmol trolox per litre and kilogram of sample, respectively. All determinations were performed using a Biotek microplate spectrophotometer (Biotek Instruments, Power Wave XS, Bad Friedrichshall, Germany) equipped with Gen5 software (Ver. 1.04.5). Each measurement was performed in duplicate.

2.7.1. TEAC

A phosphate buffer was prepared by blending 812 mL of a $Na_2HPO_4{*}2H_2O$ solution (66 mmol/L) with 182 mL KH_2PO_4 solution (66 mmol/L) and 8.8 g sodium chloride. For the daily preparation of the ABTS radical solution, 0.5 mL of ABTS solution (20 mmol/L) in phosphate buffer were mixed with 100 mL ABAP solution (2.5 mmol/L in phosphate buffer) and heated at 60 °C for 15 min in a water bath. For the determination of antioxidant capacity, 40 μL of the diluted samples were blended with 200 μL of reagent in microplate cuvettes, and the absorbance was measured after 6 min at 734 nm (van den Berg, Haenen, van den Berg, & Bast, 1999).

2.7.2. FRAP

Antioxidant activity was determined according to a procedure adopted from Benzie and Strain (1996). The FRAP reagent contained 2.5 mL of a TPTZ solution (10 mmol/L) in hydrochloric acid (40 mmol/L) and 2.5 mL of a $FeCl_3$ solution (20 mmol/L) blended with 25 mL of an acetate buffer (0.3 mol/L, pH 3.6). Aliquots of 20 μL of diluted samples were combined with 150 μL of FRAP reagent. After 4 min the absorbance of the reaction mixture was measured at 593 nm.

2.8. Photometric quantification of total phenolics

The quantification was based on a method published by Singleton, Orthofer, and Lamuela-Raventós (1999). Briefly, 60 μL of the Folin-Ciocalteu reagent were added to 50 μL of diluted pomegranate extracts and juices in microplate cuvettes. After 3 min 80 μL of a sodium carbonate solution (75 g/L) was added. The mixture was thoroughly shaken and incubated for 60 min at 20 °C in the dark. The absorbance at 720 nm was determined using a Biotek microplate spectrophotometer (Biotek Instruments, Power Wave XS, Bad Friedrichshall, Germany) equipped with Gen5 software (Ver. 1.04.5). The total phenolic contents were calculated using a calibration prepared with gallic acid (calibration range 1–200 mg/L).

2.9. Statistical analysis

Tukey's studentised range (HSD) test, using SAS software (Ver. 9.1., SAS Institute Inc., Cary, NC (USA)) was performed to determine significance of differences. The significance was determined using least significant difference (LSD) ($\alpha = 0.05$).

3. Results and discussion

3.1. Fractionation and HPLC separation of pomegranate polyphenolics

In this study, a stationary phase with hydrophilic endcapping, which has been demonstrated to be suitable for the separation of phenolic compounds from different matrices such as apple (Schieber, Keller, & Carle, 2001), mango (Schieber, Berardini, & Carle, 2003), Kiwi (Dawes & Keene, 1999), black carrots (Kammerer, Carle, & Schieber, 2004a), grape pomace (Kammerer, Claus, Carle, Schieber, 2004b) and different fruit juices (Amakura, Okada, Tsuji, & Tonogai, 2000), was used for the analysis of hydrolysable tannins, such as ellagitannins and gallotannins, as well as phenolic acids and anthocyanins from pomegranate. Since the availability of reference substances was limited, HPLC coupled to mass spectrometry proved to be extremely helpful for peak assignment and further characterisation of individual compounds.

3.2. Characterisation of phenolic compounds by LC–MS^n

3.2.1. Anthocyanins

Pomegranate anthocyanins were characterised by comparison of their UV–Vis spectra, retention times and mass spectra with those of reference substances. Fig. 1 shows a typical HPLC chromatogram of pomegranate juice (variant 1) anthocyanins recorded at 520 nm. The retention times, UV/Vis and mass spectral characteristics as well as peak assignments for all compounds are specified in Table 1. The anthocyanin profiles of the pomegranate juices and the peel extract were similar; however, peak areas of individual compounds varied. The most polar anthocyanin among the pomegranate pigments was delphinidin 3,5-diglucoside (compound **A1**), which eluted first, followed by cyanidin 3,5-diglucoside (**A2**), pelargonidin 3,5-diglucoside (**A3**), delphinidin 3-glucoside (**A4**), a cyanidin–pentoside–hexoside (**A5**), cyanidin 3-glucoside (**A6**), cyanidin 3-rutinoside (**A7**), pelargonidin 3-glucoside (**A8**) and a cyanidin-pentoside (**A9**). Monoglycosylated anthocyanins are less polar than diglycosylated compounds. Accordingly, the latter were eluted first from the HPLC column. The anthocyanins revealed the typical mass spectrometric behavior in ESI(+)-experiments, i.e. they showed M^+ ions in the MS^1 experiments and the sequential loss of their saccharide moieties, releasing the aglycones in the MS^2 and MS^3 experiments (Table 1). Only six compounds, **A1**–**A4**, **A6** and **A8**, have previously been reported in pomegranates (Gil, Garcia-Viguera, Artés, & Tomás-Barberán, 1995, Herndez et al., 1999), and these anthocyanins were shown to significantly differ in their contents in different cultivars (Algihourchi & Barzegar, 2008). To our knowledge compound **A5** has not been reported in *Punica granatum* L. so far. Furthermore, compounds **A7** and **A9** have not generally been described in the pomegranate literature, thus demonstrating that the pigment profile of pomegranates may be much more complex. The aforementioned compounds

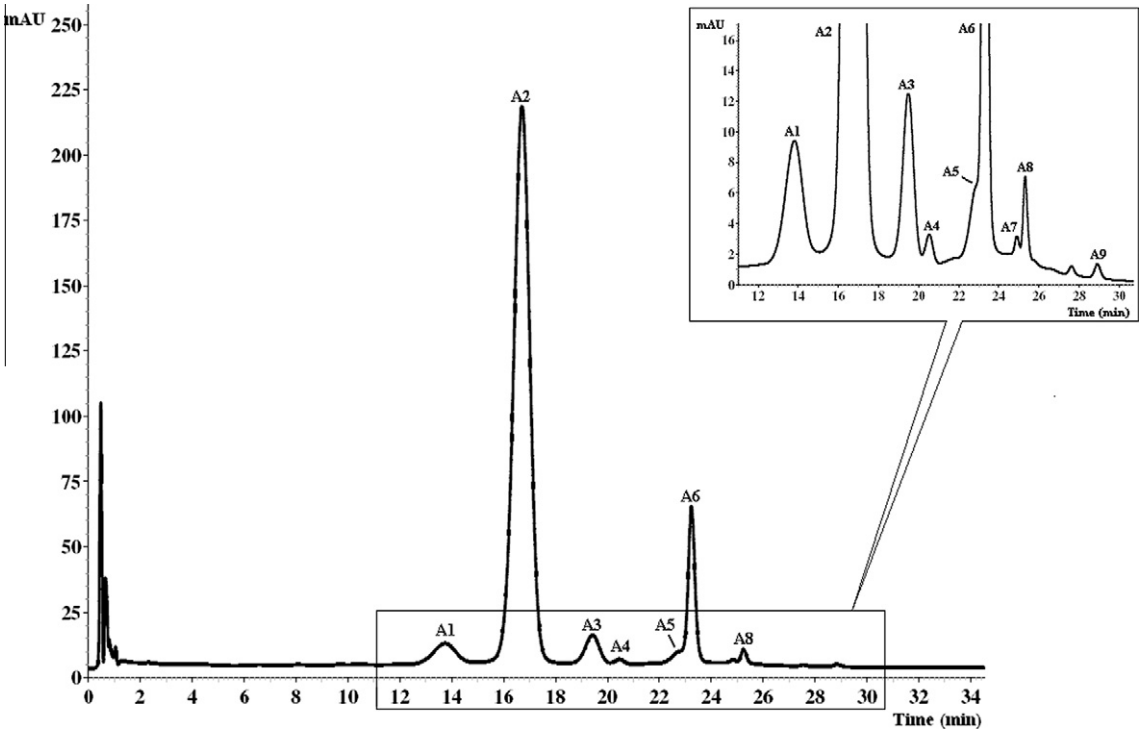

Fig. 1. Separation of anthocyanins in pomegranate juice (variant 1) by HPLC (520 nm). For peak assignment see Table 1.

Table 1
Retention times, UV/Vis spectra and characteristic ions of anthocyanin compounds of pomegranate.[a,b]

Compd	Assignment	rt (min)	HPLC-DAD λ_{max} (nm)	$[M]^+$ m/z	HPLC–ESI(+)-MS^n experiment m/z (% base peak)	Occurrence				
						Juice variant			p	m
						1	2	3		
Anthocyanins										
A1	Del-3,5-diglc	15.0	519, 277	627	– MS^2 [627]: 465 (100), 303 (72), 304 (15) – MS^3 [627 → 465]: 303 (100)	+	+	+	+	–
A2	Cya-3,5-diglc	18.0	513, 277	611	– MS^2 [611]: 287 (100), 449 (82) – MS^3 [611 → 449]: 287 (100), 288 (20)	+	+	+	+	–
A3	Pel-3,5-diglc	20.7	499, 274	595	– MS^2 [595]: 433 (100), 434 (59), 271 (48) – MS^3 [595 → 433]: 271 (100)	+	+	+	+	–
A4	Del-3-glc	21.7	522, 341, 277	465	– MS^2 [465]: 303 (100)	+	+	+	+	–
A5	Cya-pent-hex	24.1	516, 273	581	– MS^2 [581]: 449 (100), 287 (77), 419 (29) – MS^3 [581 → 449]: 287 (100)	+	+	+	+	–
A6	Cya-glc	24.5	516, 323, 280	449	– MS^2 [449]: 287 (100)	+	+	+	+	–
A7	Cya-rut	26.1	503, 423, 327, 274	595	– MS^2 [595]: 287 (100), 449 (39), 450 (26) – MS^3[595 → 287]: 149 (100)	+	+	+	+	–
A8	Pel-glc	26.5	503, 427, 329, 274	433	–MS^2 [433]: 271 (100)	+	+	+	+	–
A9	Cya-pent	30.1	515, 278	419	– MS^2 [419]: 287 (100)	+	+	+	+	–

[a] Abbreviations: compd, compound; cya, cyanidin; del, delphinidin; glc, glucoside; m, mesocarp; p, peel; pel, pelargonidin; pent, pentoside; rt, retention time; rut, rutinoside.
[b] +, detected; –, not detected.

may have been either overlooked in previous studies due to their low amounts, or their occurrence may be cultivar-dependent.

The separation of non-anthocyanin polyphenolics in pomegranate juice (variant 1) is illustrated in Figs. 2 and 3. Among the numerous phenolic compounds, 38 were characterised as hydrolyzable tannins, such as gallotannins (group B), ellagitannins (group C) and gallagyl esters (group D), as well as hydroxybenzoic acids (group E), hydroxycinnamic acids (group F) and one dihydroflavonol (group G) based on their UV and mass spectrometric data, which are specified in Table 2.

3.2.2. Gallotannins

The gallotannins (group B) showed nearly identical UV spectra, which are similar to that of gallic acid (λ_{max} = 269 nm). Compound **B1** revelaed an $[M-H]^-$ ion at m/z 331 and produced fragment ions at m/z 169 (MS^2), indicating the loss of a hexose moiety and m/z 125 (MS^3) typical for gallic acid and its loss of the carboxylic function upon collision-induced dissociation (CID). Therefore, this compound was identified as monogalloyl-hexoside, which was in accordance with previous results of Gil et al. (2000). Compound **B2** (m/z 483) was assigned to a digalloylhexoside. In addition to **B1**, it produced fragments at m/z 331 and m/z 313, which are due to the loss of a gallic acid moiety (152 Da) and a further loss of water (18 Da). Two compounds with m/z 483 in the MS^1 experiment were detected after 6.4 and 45.9 min indicating the occurrence of isomeric structures which significantly differ in their elution behaviour. The galloylhexoside was only identified in juice variants 1 and 3, whereas at least one of the isomers of **B2** was found in all samples investigated.

3.2.3. Ellagitannins

By acid hydrolysis of the samples the presence of ellagic acid derivatives was verified, because the amounts of ellagic acid in

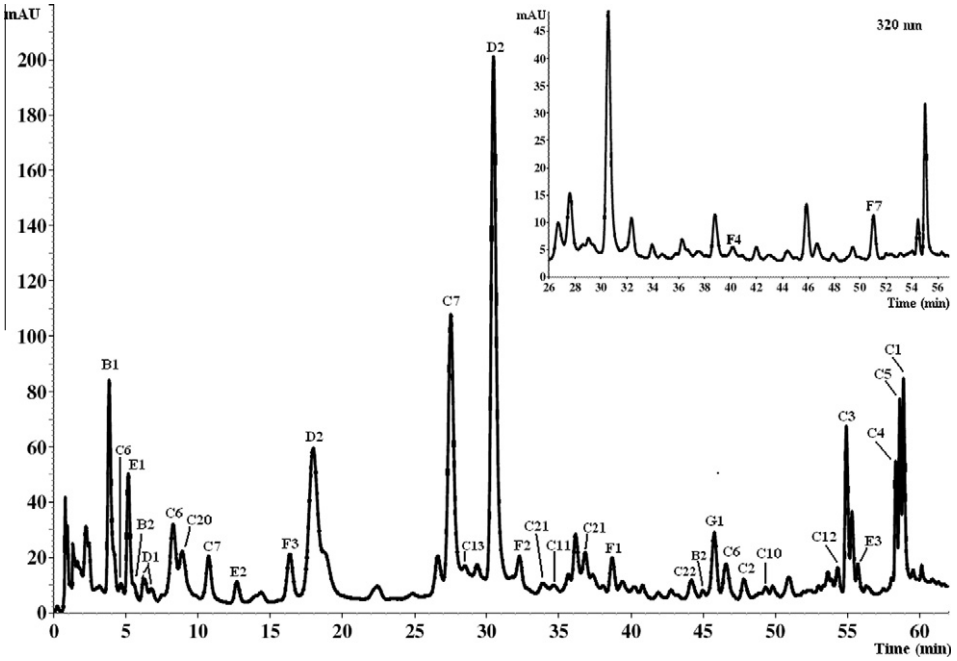

Fig. 2. Separation of non-anthocyanin phenolic compounds in pomegranate juice (variant 1) by HPLC (fraction I, 280 and 320 nm). For peak assignment see Table 2.

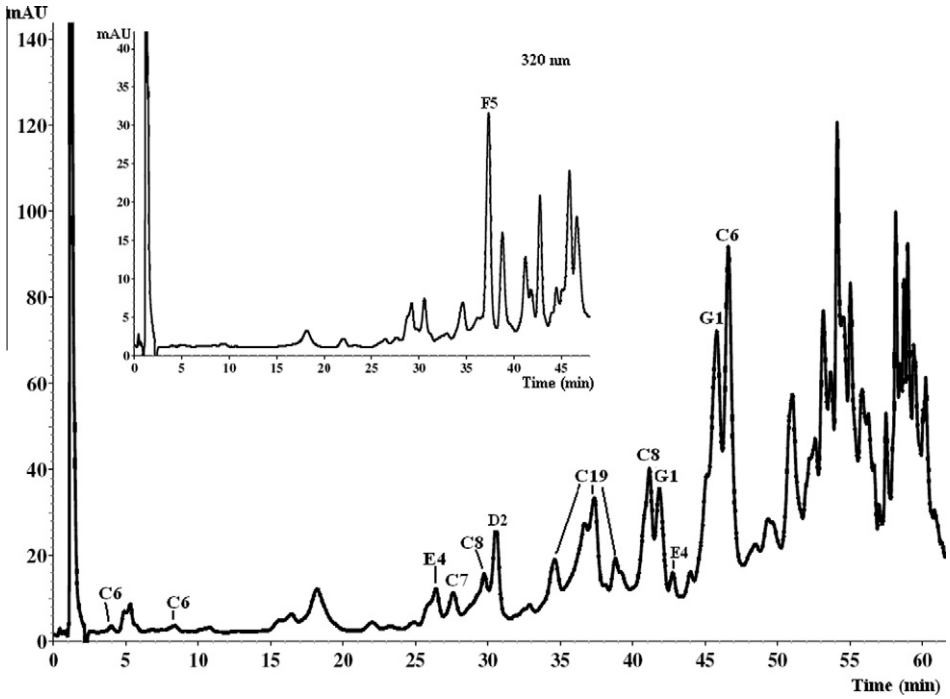

Fig. 3. Separation of non-anthocyanin phenolic compounds in pomegranate juice (variant 1) by HPLC (fraction II, 280 and 320 nm). For peak assignment see Table 2.

the samples were markedly increased upon hydrolysis. The aglycone showed the same $[M-H]^-$ ion at m/z 301 like quercetin. However, ellagic acid produced fragments at m/z 258, 229 and 185 in the MS^2 experiment, whereas quercetin generates fragments at m/z 179 and 151 upon CID (Kammerer, Claus, Carle, Schieber, 2004b; Mullen, Yokato, Lean, & Crozier, 2003). Furthermore, both compounds significantly differ in their UV/Vis spectra (λ_{max}; ellagic acid: 275 and 367 nm; quercetin: 252, 286, and 372 nm), thus allowing their unambiguous differentiation. Ellagic acid has previously been reported for pomegranate husk and juices (Gil et al., 2000; Seeram, Lee, Hardy, & Heber, 2005).

Two compounds (**C2**) with m/z 481 in the MS^1 experiment were detected. The compound eluted after 3.1 min produced a fragment at m/z 301 indicating the release of an ellagic acid moiety. This compound was detected in the mesocarp and peel. The second compound eluting after 48.8 min was identified in juice 3 made from isolated arils. The loss of water provided a fragment at m/z 463 (ellagic acid-hexoside; **C3**), which is typical of ellagitannins. Both compounds were tentatively identified as hexahydroxydiphenoyl-hexoside (HHDP-hexoside, Fig. 4). The different retention times and fragmentation patterns indicated the occurrence of two isomeric compounds with different linkage type. HHDP-hexoside has previously been characterised in the bark of pomegranate trees by NMR spectroscopy (Tanaka, Nonaka, & Nishika, 1986b). In addition to ellagic acid hexoside (m/z 463) further monoglycosylated ellagic acid derivatives were observed in pomegranate, such as an ellagic acid-pentoside (m/z 433; **C4**) and deoxyhexoside (m/z 447; **C5**), all of them showing the typical fragments of ellagic acid (m/z 301; **C1**), frequently based on the stabilisation possibilities of that structure for a quinoidic system (m/z 299) and a distonic radical (m/z 300), which contain both a radical and an ionic site. The fragment at m/z 271 was formed by the loss of a carbonyl moiety from the oxidised structure, which is in accordance with previous reports (Nawar, Marzouk, Nigge, & Linscheid, 1997). Ellagitannins **C3**–**C5** have been detected previously during a large scale purification of pomegranate husk polyphenols (Seeram et al., 2005).

The ellagic acid hexoside moiety was also observed as a fragment of compound **C6**, showing an $[M-H]^-$ ion at m/z 633 and fragments at m/z 615 and 301 in the MS^2 experiment, which are typical for the loss of water and ellagic acid. Compound **C6** was identified as galloyl-HHDP-hexoside (Fig. 4), and three isomeric compounds with similar fragmentation patterns were observed (Table 2, Figs. 2 and 3). These compounds may be assigned either to corilagin, strictinin or punicacortein A or B, depending on the linkage type. While corilagin is linked via the 3,5-position of the glycosyl unit and, stricitin has a 4,5-linkage. In contrast, the punicacorteins are linked at the 2,3-position of the glycosyl unit and an additional C-glycosidic bond (Tanaka et al., 1986b). Thus, the compounds only differ in the position of the galloyl moiety, however, based on the data of the present study, a further assignment was impossible.

Compounds **C1**–**C6** were released as fragments in MS^2 and MS^3 experiments of further pomegranate ellagitannins described below. Compound **C7** exhibited an $[M-H]^-$ ion at m/z 783. The loss of water and ellagic acid in the MS^2 experiment produced fragments at m/z 765 and m/z 481, respectively. Based on this fragmentation pathway and the occurrence of further typical fragments as described above, compound **C7** was identified as bis-HHDP-hexoside (pedunculagin I, Fig. 4a) (Fig. 5). UV- and mass spectrometric data are shown in Fig. 6. Compound **C7** occurred in three isomeric forms as can be deduced from the retention times given in Table 2, and the isomers differed in their fragmentation pathways (Okuda, Yoshida, Ashida, & Yazaki, 1983; Seikel & Hillis, 1970). Compound **C8** was characterised as digalloyl-HHDP-hexoside (pedunculagin II). This assignment is based on its $[M-H]^-$ ion at m/z 785 and the release of typical ellagitannin and gallotannin fragments at m/z 483 (digalloylhexoside), 301 (ellagic acid) and 633 (galloyl-HHDP-hexoside). Each of the two different retention times corresponded to an isomeric structure, also differing in their fragmentation patterns. Compounds **C7** and **C8** were first described in the bark and leaves of *P. granatum* L. (Tanaka et al., 1986b; Hussein, Barakat, Merfort, & Nawar, 1997). Furthermore, compound **C9** showed an $[M-H]^-$ ion at m/z 935 and typical fragments at m/z 633 and 615

Table 2
Retention times, UV/Vis spectra and characteristic ions of non-anthocyanin phenolic compounds of pomegranate.[a,b]

Compd	Assignment	rt (min)	HPLC-DAD λ_{max} (nm)	$[M-H]^-$ m/z	HPLC–ESI(−)-MS^n experiment m/z (% base peak)	Occurrence				
						Juice variant			p	m
						1	2	3		
Hydrolysable tannins										
	Gallotannins									
B1	Galloyl-hex	4.8	375, 266	331	– MS^2 [331]: 169 (100), 271 (64), 331 (38) – MS^3 [331 → 169]: 125 (100)	+	–	+	–	–
B2	Digalloyl-hex	6.4	363, 264	483	– MS^2 [483]: 331 (100), 169 (74) – MS^3 [483 → 331]: 169 (100), 193 (85), 125 (73)	+	–	–	–	–
		45.9	366, 258		– MS^2 [483]: 313 (100), 169 (35) – MS^3 [483 → 313]: 169 (100), 125 (52)	+	+	+	+	+
	Ellagitannins									
C1	Ellagic acid	59.8	367, 275	301	– MS^2 [301]: 301 (100), 229 (62), 185 (39) – MS^2 [301]: 258 (100), 174 (66), 185 (49), 301 (20), 257 (17)	+	+	+	+	+
C2	HHDP-hex	3.1	267	481	– MS^2 [481]: 301 (100), 302 (12)	–	–	–	+	+
		48.8	267		– MS^2 [481]: 463 (100), 315 (93), 345 (66) – MS^3 [481 → 463]: 297 (100), 165 (43)	–	–	+	–	–
C3	Ellagic acid-hex	55.7	361, 252	463	– MS^2 [463]: 301 (100), 300 (33), 302 (12) – MS^3 [463 → 301]: 271 (100), 301(49)	+	+	+	+	+
C4	Ellagic acid-pent	59.3	359, 255	433	– MS^2 [433]: 301 (100), 300 (97) – MS^3 [433 → 301]: 271 (100)	+	+	+	+	+
C5	Ellagic acid-deoxyhex	59.6	360	447	– MS^2 [447]: 300 (100), 301 (80), 302 (17)	+	+	+	–	+
C6	Galloyl-HHDP-hex	4.7	365, 266	633	– MS^2 [633]: 301 (100), 249 (28), 302 (27), 463 (5)	+	+	–	+	+
		9.2	330, 260		– MS^2 [633]: 301 (100), 615 (31), 249 (24) – MS^3 [633 → 301]: 301 (100), 185 (34)	+	–	–	–	–
		47.6	365, 256		– MS^2 [633]: 301 (100), 463 (26), 302 (20) – MS^3 [633 → 301]: 301 (100), 229 (39)	+ +*	+ +*	+	+	+ +*
C7	bis-HHDP-hex (pedunculagin I)	5.5	377, 253	783	– MS^2 [783]: 481 (100), 301 (62), 375 (27) – MS^3 [783 → 481]: 275 (100), 301 (74)	–	+	–	+	+
		11.5	375, 258		– MS^2 [783]: 301 (100), 481 (55), 482 (26) – MS^3 [783 → 481]: 301 (100), 275 (31)	+	+	–	+	+
		28.3	375, 259		– MS^2 [783]: 765 (100), 766 (26) – MS^3 [783 → 765]: 747 (100), 613 (54), 275 (55), 463 (25)	+ +*	+ +*	+	–	–
C8	Digalloyl-HHDP-hex (pedunculagin II)	30.6	272	785	– MS^2 [785]: 633 (100), 483 (62), 765 (42) – MS^3 [785 → 633]: 463 (100)	+*	+*	+*	+*	+*
		42.0	274		– MS^2 [785]: 483 (100), 301 (35), 633 (28) – MS^3 [785 → 483]: 169 (100), 193 (67), 170 (26)	+*	+*	+*	+*	+*
C9	Galloyl-bis-HHDP-hex (casuarinin)	35.0	367, 265	935	– MS^2 [935]: 633 (100), 615 (22), 659 (16), 571 (16) – MS^3 [935 → 633]: 571 (100), 615 (46), 481 (41)	–	–	–	+	+
C10	Ellagic acid der	50.7	370, 254	441	– MS^2 [441]: 397 (100), 398 (21), 300 (4) – MS^3 [441 → 397]: 380 (100), 379 (81), 299 (60)	+	–	–	+	+
C11	Ellagic acid der	35.6	272	443	– MS^2 [443]: 301 (100), 162 (36), 219 (34)	+	–	+	–	–
C12	Ellagic acid dihex	55.3	319, 286	625	– MS^2 [625]: 301 (100), 463 (37)	+	–	–	–	–
C13	Valoneic acid bilactone	28.2	373, 254	469	– MS^2 [469]: 425 (100) – MS^3 [469 → 425]: 407 (100), 300 (61), 301 (32)	+	+	–	+	+
		52.2	364, 256		– MS^2 [469]: 425 (100), 426 (54) – MS^3 [469 → 425]: 301 (100), 300 (93), 299 (65)	–	+		+	+
C14	Ellagic acid der	30.7	260	392	– MS^2 [392]: 316 (100), 324 (44) – MS^3 [392 → 316]: 169 (100), 307 (66), 301 (50)	–	–	+	–	–
C15	Flavogalloyl-HHDP-gluconic acid (lagerstannin B)	56.8	392, 258	949	– MS^2 [949]: 905 (100), 906 (31) – MS^3 [949 → 905]: 615 (100), 302 (73), 301 (59)	–	–	–	+	+
C16	Lagerstannin B der	36.2	378, 259	967	– MS^2 [967]: 905 (100), 851 (69), 825 (71), 949 (42) – MS^3 [967 → 905]: 433 (100), 735 (61), 738 (50), 301 (39)	–	–	–	+	–
C17	Galloyl-HHDP-DHHDP-hex (granatin B)	54.0	365, 274	951	– MS^2 [951]: 933 (100), 934 (46), 915 (12) – MS^3 [951 → 933]: 301 (100), 915 (52), 613 (41), 463 (18)	–	+*	–	+*	+*
C18	Castalagin der	56.7	275	965	– MS^2 [965]: 933 (100), 934 (28) – MS^3 [951 → 933]: 301 (100), 897 (69), 613 (65), 915 (20)	–	+*	–	+*	+*
C19	Ellagic acid der	37.6–39.9	265	799	– MS^2 [799]: 479 (100), 781 (46), 301 (36), 299 (13), 331 (12) – MS^3 [799 → 479]: 271 (100), 391 (83), 451 (31), 263 (28)	+	+*	–	+*	+*

(*continued on next page*)

Table 2 (*continued*)

Compd	Assignment	rt (min)	HPLC-DAD λ_{max} (nm)	$[M-H]^-$ m/z	HPLC–ESI(−)-MS^n experiment m/z (% base peak)	Occurrence				
						Juice variant			*p*	*m*
						1	2	3		
C20	Galloyl-HHDP-gluc (lagerstannin C)	3.2	250	649	– MS^2 [649]: 301 (100), 497 (69) – MS^3 [649 → 301]: 185 (100)	–	+	–	+	+
		11.6	257		– MS^2 [649]: 497 (100), 301 (81), 498 (29) – MS^3 [649 → 497]: 301 (100)	+	+	–	+	+
C21	Digalloyl-HHDP-gluc (punigluconin)	35.0	375, 268	801	– MS^2 [801]: 649 (100), 347 (84), 348 (65) – MS^3 [801 → 649]: 301 (100), 195 (60)	–	–	–	+	–
		37.1	370, 261		– MS^2 [801]: 649 (100), 499 (32), 301 (30) – MS^3 [801 → 649]: 301 (100), 302 (53), 497 (40)	+	+	–	–	+
		39.6	275, 264		– MS^2 [801]: 499 (100), 649 (97), 301 (59) – MS^3 [801 → 649]: 347 (100), 301 (79), 302 (80)	–	+	–	+	+
C22	Brevifolin carboxylic acid	44.1	351, 274	291	– MS^2 [291]: 247 (100), 248 (16) – MS^3 [291 → 247]: 203 (100), 204 (50), 177 (18)	+	+	–	+	+
	Gallagyl esters									
D1	Gallagyl-hex (punicalin)	7.2–7.6	371, 262	781	– MS^2 [781]: 601 (100), 602 (28), 721 (16) – MS^3 [781 → 601]: 243 (100), 601 (51), 299 (29)	+	+	–	+	–
D2	HHDP-gallagyl-hex (punicalagin)	18.9	378, 258	1083	– MS^2 [541]: 601 (100), 575 (26), 302 (20), 781 (20) – MS^3 [541 → 601]: 271 (100), 583 (32), 272 (29)	+	+	–	+	+
		31.5	378, 258		– MS^2 [541]: 601 (100), 603 (35), 781 (28) – MS^3 [541 → 601]: 299 (100), 501 (21)	+	+	+	+	+
Hydroxybenzoic acids										
E1	Gallic acid	6.1	269	169	– MS^2 [169]: 125 (100)	+	+*	+	+	+
E2	Protocatechuic acid	13.6	294	153	– MS^2 [153]: 109 (100)	+	–	+	–	–
E3	Protocatechuic acid-der	56.3	269	425	– MS^2 [425]: 197 (100), 153 (32) – MS^3 [425 → 197]: 153 (100), 109 (4)	+	–	+	–	–
E4	Vanillic acid-hex	23.5	292, 258	329	– MS^2 [329]: 167 (100), 168 (21), 239 (45) – MS^3 [329 → 167]: 108 (100), 123 (62), 152 (56)	–	+*	+	–	–
		43.4	292, 262		– MS^2 [329]: 167 (100), 209 (80), 210 (12) – MS^3 [329 → 167]: 152 (100), 123 (74), 108 (31)	+*	–	+*	–	–
Hydroxycinnamic acids										
F1	Caffeic acid hex	30.1	293	341	– MS^2 [341]: 179 (100), 161 (25), 135 (10) – MS^3 [341 → 179]: 135 (100)	–	–	+*	–	–
		39.7	283		– MS^2 [341]: 179 (100), 135 (25) – MS^3 [341 → 179]: 135 (100)	+	–	–	–	–
		41.2	290		– MS^2 [341]: 179 (100), 135 (21) – MS^3 [341 → 179]: 135 (100)	+	–	+	–	–
F2	Caffeic acid hex der	33.3	293	451	– MS^2 [451]: 341 (100), 342 (11) – MS^2 [451 → 341]: 169 (100), 179 (96), 151 (82), 341 (57)	+	–	+	–	–
F3	Caffeic acid der	17.3	260	299	– MS^2 [299]: 137 (100), 179 (53), 239 (45) – MS^3 [299 → 137]: 93 (100)	+	+	+	–	–
F4	5-*O*-Caffeoylquinic acid	40.9	325	353	– MS^2 [353]: 191 (100) – MS^3 [353 → 191]: 127 (100)	+	–	–	–	–
		38.2	282	325	– MS^2 [325]: 145 (100), 163 (88), 187 (42) – MS^3 [325 → 163]: 119 (100)	+*	–	+*	–	–
		39.7	290		– MS^2 [325]: 163 (100), 145 (90), 117 (11), 187 (36) – MS^3 [325 → 163]: 119 (100)	–	–	+*	–	–
F6	Ferulic acid-hex	43.7	326, 290	355	– MS^2 [355]: 193 (100), 175 (65), 217 (55), 236 (22) – MS^3 [355 → 193]: 134 (100), 149 (59), 178 (9), 176 (6)	–	–	+	–	–
F7	Coumaric acid	51.9	304	163	– MS^2 [163]: 119 (100)	+	–	+	–	–
Dihydroflavonol										
G1	Dihydrokaempferol-hex	41.9	292	449	– MS^2 [449]: 287 (100), 259 (80), 269 (47), 288 (22) – MS^3 [449 → 287]: 259 (100), 260 (11), 243 (2)	–	–	+	–	–
		44.4	292		– MS^2 [449]: 287 (100), 259 (75), 269 (29), 179 (27) – MS^3 [449 → 287]: 259 (100), 243 (31), 201 (26)	+ +*	+	+	–	–

[a] Abbreviations: compd, compound; cya, cyanidin; del, delphinidin; deoxyhex, deoxyhexoside; der, derivative; glc, glucoside; hex, hexoside; HHDP,hexahydroxydiphenoyl; m, mesocarp; p, peel; pel, pelargonidin; pent, pentoside; rt, retention time; rut, rutinoside.

[b] +, fraction I; +*, fraction II; – not detected.

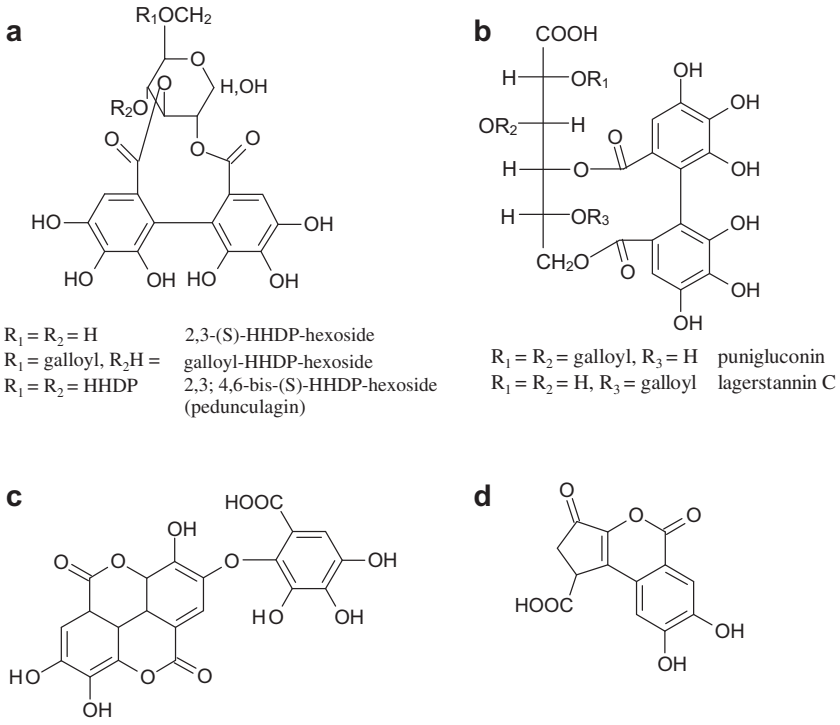

Fig. 4. Structures of ellagitannins with a glycosyl (a) and a gluconic acid core (b), valoneic acid bilactone (c) and brevifolin carboxylic acid (d).

(galloyl-HHDP-hexoside and the dehydrated derivative) as well as *m*/*z* 481 (HHDP-hexoside) and was identified as galloyl-bis-HHDP-hexoside (casuaricitin) (Tanaka et al., 1986b; Okuda et al., 1983; Karvela, Makris, Kefalas, & Moutounet, 2008).

Compound **C12** (*m*/*z* 625) also yielded a fragment at *m*/*z* 463 (ellagic acid hexoside) and 301 (ellagic acid) and showed a loss of 162 Da indicating a second hexose moiety. Therefore, compound **C12** was assigned to an ellagic acid-dihexoside.

An $[M-H]^-$ ion at *m*/*z* 469 was observed for the isomeric compound **C13**, producing fragment ions at *m*/*z* 425 and 407, thus indicating the loss of a carboxyl group and of water. Additionally, typical fragments of ellagic acid (*m*/*z* 300 and 301) were observed for both isomers with retention times of 28.2 and 52.2 min. This fragmentation pattern and the UV/Vis absorption spectrum were consistent with previous reports (Nawar et al., 1997; Mämmelä, Savolainen, Lindroos, Kangas, & Vartiainen, 2000), and compound **C13** was therefore assigned to the valoneic acid bilactone isomers (Fig. 4c). As previously reported, this compound also showed dimer formation in the ionisation source (Nawar et al., 1997). However, to our knowledge valoneic acid bilactone has not yet been reported in *P. granatum* L. so far.

Compounds **C10** (*m*/*z* 441), **C11** (*m*/*z* 443) and **C14** (*m*/*z* 392) revealed typical ellagic acid fragments (*m*/*z* 301, 300 and 299) and, thus, were assumed to be ellagic acid derivatives. However, further information for a more detailed characterisation of these three compounds was not obtained.

In addition, ellagitannins with a gluconic acid core were also found in pomegranates. Among these, compound **C20** was identified as galloyl-HHDP-gluconic acid (lagerstannin C, Fig. 4b) (Tanaka et al., 1992). Its $[M-H]^-$ ion at *m*/*z* 649 released fragments at *m*/*z* 497 and 301, resulting from the loss of gallic acid (releasing HHDP-gluconic acid) and ellagic acid, respectively. Galloyl-HHDP-gluconic acid (*m*/*z* 649) also formed part of compound **C21** exhibiting an $[M-H]^-$ ion at *m*/*z* 801. The difference of 152 Da indicated another gallic acid moiety. Further fragments at *m*/*z* 348 and 497 resulted from the loss of ellagic acid and gallic acid. Accordingly, this compound (**C21**) was identified as digalloyl-HHDP-gluconic acid (punigluconin) (Tanaka et al., 1992), which has previously been isolated and characterised in the bark of *P. granatum* L. (Tanaka et al., 1986b).

Compound **C15** produced an $[M-H]^-$ ion at *m*/*z* 949 and the aforementioned fragments at *m*/*z* 615 and 301. These signals were assigned to flavogalloyl-HHDP-gluconic acid (lagerstannin B) (Tanaka et al., 1992). Compound **C16** (*m*/*z* 967) revealed similar fragments and an additional fragment at *m*/*z* 949. Accordingly, this component was assumed to be a hydroxylated derivative of lagerstannin B.

Compound **C19** produced an $[M-H]^-$ ion at *m*/*z* 799 and fragments at *m*/*z* 781 (loss of water) and at *m*/*z* 479 (loss of ellagic acid). Furthermore, the established fragments of ellagic acid were confirmed. This compound may either be attributed to granatin A (HHDP-DHHDP-hexoside) or lagerstannin A (bis-HHDP-gluconic acid). Unfortunately, a more detailed characterisation of this ellagitannin was impossible.

Compound **C17** exhibited an $[M-H]^-$ ion at *m*/*z* 951 and compound **C18** at *m*/*z* 965. Both compounds produced fragments at *m*/*z* 933 and 934 in the MS^2 experiment and the typical fragment pointing to ellagic acid (*m*/*z* 301) in the MS^3 experiment. Furthermore, fragments at *m*/*z* 915 were obtained from the loss of water from the major fragment (*m*/*z* 933) and the ion at *m*/*z* 897 by dehydration. Furthermore, the fragment at *m*/*z* 613 resulted from additional losses of ellagic acid and water from the prominent fragment. This fragment (*m*/*z* 933) is typical for castalagin/vescalagin or galloyl-gallagyl-hexoside (galloylpunicalin, pedunculagin III). However, the characteristic gallagyl-fragment at *m*/*z* 601 was not detected. Furthermore, castalagin/vescalagin exhibited molecular masses 18 Da lower than compound **C17**. Based on these results, compound **C17** was identified as granatin B (galloyl-HHDP-DHHDP-hexoside), which forms part of type III-tannins (dehydroellagitannins) (Okuda et al., 2000). Granatin A and B were first identified as the major components of pomegranate leaves (Tanaka, 1985).

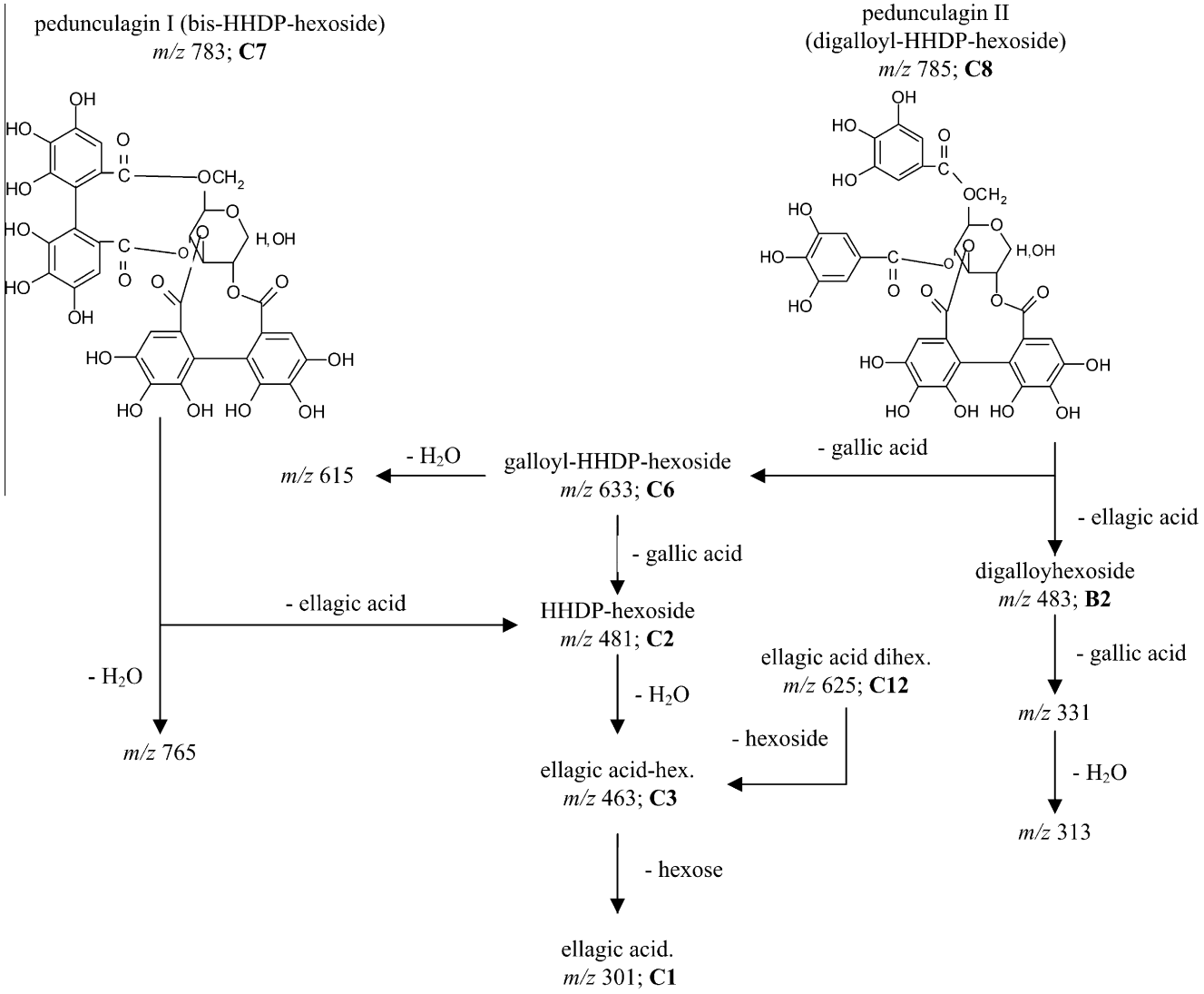

Fig. 5. Postulated fragmentation pathways of bis-HHDP-hexoside and digalloyl-HHDP-hexoside.

Compound **C18** (*m*/*z* 965) may be a derivative of castalagin/vescalagin as well, because the ions at *m*/*z* 915 and 897 indicate fragmentation of these core molecules releasing water.

Compound **C22** was characterised by an $[M-H]^-$ ion at *m*/*z* 291 and fragments, which are due to the loss of carboxyl moieties (*m*/*z* 247 and 203). The MS and UV/Vis data were in agreement with those previously reported for brevifolin carboxylic acid (Fig. 4d) in pomegranate leaves (He & Xia, 2007; Hussein et al., 1997; Nawar, Hussein, & Merfort, 1994). To the best of our knowledge brevifolin carboxylic acid has not yet been reported in pomegranate fruits.

3.2.3.1. Gallagic acid and gallagyl esters. Punicalagin is the major phenolic compound in pomegranate (**D2**; 2,3-HHDP-4,6-gallagyl-glucoside) and has already been well characterised (Tanaka et al., 1986a). As commonly known for the ellagitannins (Mullen et al., 2003; Seeram, Lee, Scheuller, & Heber, 2006), punicalagin was also detected as doubly charged ion species displaying an $[M-2H]^{2-}$ ion at *m*/*z* 541, which is equivalent to a molecular weight of 1084 Da. The fragment at *m*/*z* 601 in the MS^2 experiment indicated the loss of a gallagic acid moiety. The fragments in the MS^3 experiment, with *m*/*z* 271 and 299 are attributed to the loss of ellagic acid from the gallagic acid moiety. Punicalagin occurs in two isomeric forms, the α- and β-anomers (Lu, Ding, & Yuan, 2008), which were confirmed in the present study as illustrated by different retention times of compound **D2**. The UV/Vis absorption spectrum showed maxima at 378 and 258 nm, which was in agreement with earlier reports of punicalagin in pomegranate (Gil et al., 2000). Furthermore, a fragment of **D2** at *m*/*z* 781 was observed equivalent to the $[M-H]^-$ ion of compound **D1**. The release of this ion was caused by the loss of ellagic acid. Compound **D1** was identified as punicalin (4,6-gallagyl-glucoside) which also revealed the loss of a gallagic acid moiety in the MS^2 experiment (*m*/*z* 601). In the MS^3 experiment fragments at *m*/*z* 299 and 300 were detected, characterising the quinoidic and radical structures of ellagic acid after the fission of the gallagic acid moiety. This compound has previously been identified by NMR spectroscopy in the bark of pomegranate trees (Tanaka et al., 1986a).

3.2.4. Hydroxybenzoic acids

Gallic acid (**E1**) and protocatechuic acid (**E2**) were identified by comparing the retention times and UV and mass spectra with those of reference substances. Compound **E3** revealed an $[M-H]^-$ ion at *m*/*z* 425 and fragments at *m*/*z* 179, 153 in the MS^2 experiment and *m*/*z* 153, 109 in the MS^3 experiment indicating a protocatechuic acid derivative. Furthermore, compound **E4**, producing an $[M-H]^-$ ion at *m*/*z* 329 and at *m*/*z* 167 in the MS^2 experiment, which is characteristic of vanillic acid, was identified as vanillic acid 4-hexoside. The UV/Vis absorption spectrum of **E4** showed only

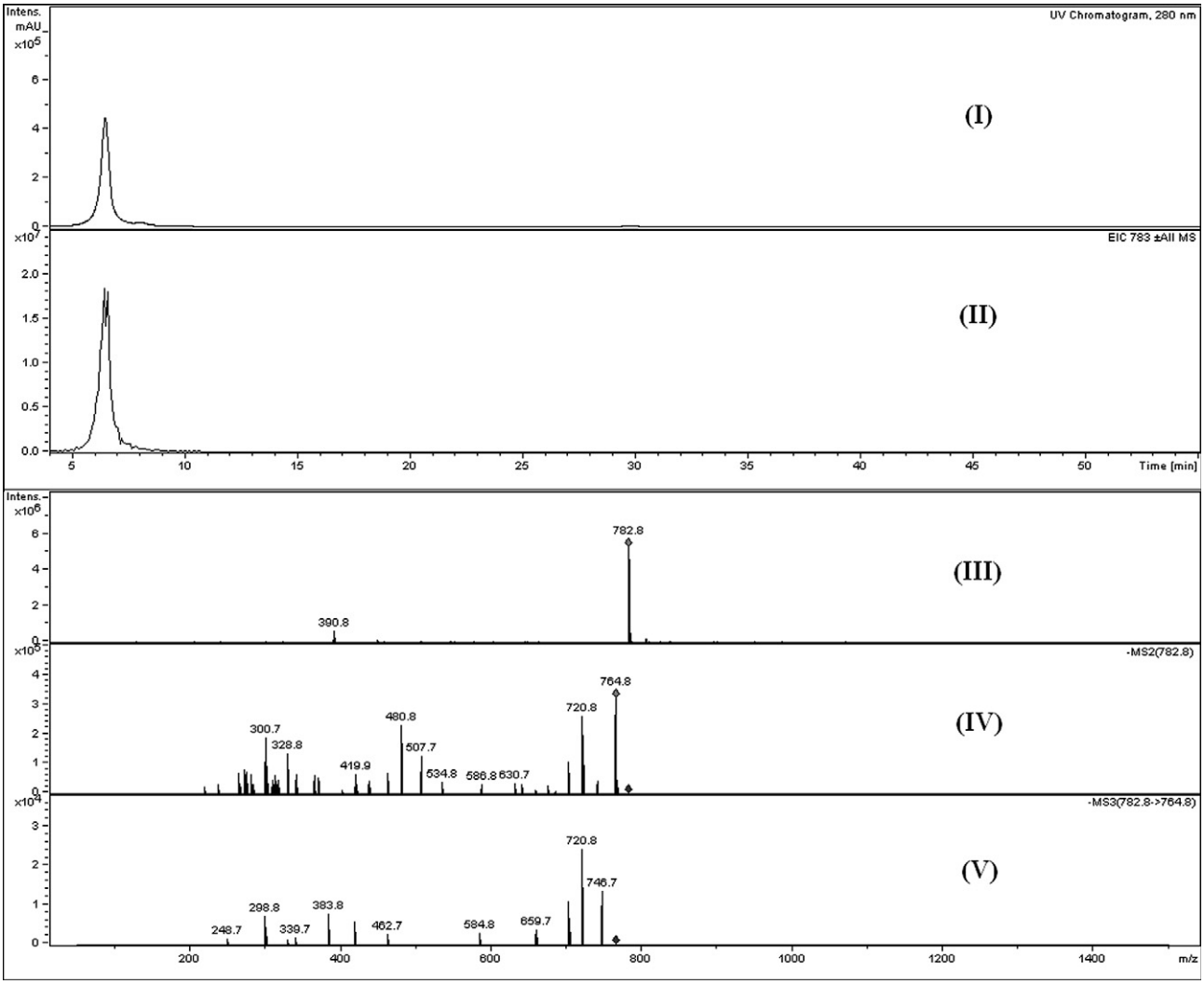

Fig. 6. HPLC–MS^n analysis of pedunculagin I (bis-HHDP-hexoside) isolated by preparative HPLC. (I) UV-chromatogram at 280 nm; (II) extracted ion chromatogram at *m*/*z* 783; (III) MS^1 spectrum of pedunculagin I; (IV) MS^2 fragment spectrum of *m*/*z* 783; (V) MS^3 fragment spectrum of *m*/*z* 765.

minor shifts of the absorption maxima as compared to vanillic acid (from 254 and 293 nm for vanillic acid to 258 and 292 nm for **E4**) which are presumably caused by the glycosylation of the hydroxyl group. These data are consistent with previous reports (Moran et al., 1998), however, to our knowledge vanillic acid 4-glucoside has not yet been reported in *P. granatum* L. so far.

3.2.5. Hydroxycinnamic acids

Among hydroxycinnamic acids (group F), compound **F1** was identified as caffeoyl hexoside (*m*/*z* 341), which showed the loss of a saccharide moiety in the MS^2 experiment (162 Da) and a partial decarboxylation of the caffeic acid moiety resulting in fragments at *m*/*z* 179 and 135. This fragmentation pattern was observed for three different compounds, characterised by the retention times specified in Table 2. This is presumably due to isomers differing in the linkage of the glycosyl moiety.

Compound **F2** revealed a $[M-H]^-$ ion at *m*/*z* 451 and a loss of 110 Da in the MS^2 experiment resulting in a fragment at *m*/*z* 341, which in turn showed a fragmentation pattern identical to compound **F1**. It was therefore concluded that compound **F2** also represents a caffeoyl hexoside conjugated with an hydrophilic compound, explaining the low retention time compared to two

Table 3
Total phenolics (Folin–Ciocalteu) [mg/L; mg/kg] and antioxidative capacity (FRAP, TEAC) [mmol/L; mmol/kg] of pomegranate juices, peels and mesocarp.[a,b]

	Juice variant			Peel	Mesocarp
	1 (mmol/L; mg/L)	2 (mmol/L; mg/L)	3 (mmol/L; mg/L)	(mmol/kg; mg/kg DM)	(mmol/kg; mg/kg DM)
FRAP	9.1 ± 0.2b	31.5 ± 1.4a	8.8 ± 0.2b	589.1 ± 34.7x	1132.7 ± 67.7x
TEAC	24.5 ± 1.0b	76.4 ± 2.0a	21.7 ± 0.0b	1361.9 ± 13.7y	2887.1 ± 6.5x
Total phenolics	2015.2 ± 21.6b	5186.0 ± 172.5a	2122.0 ± 0.0b	101856.3 ± 12810.0y	198173.3 ± 2899.7x

[a] Abbreviations: DM, dry matter.
[b] Values expressed as means of duplicate determinations ± standard error, Significant differences between values in the same line are indicated by different letters a/b and x/y ($P < 0.05$).

of the **F1** isomers. Compound **F3** (*m*/*z* 299) was classified as a caffeic acid derivative based on its prominent fragment at *m*/*z* 137 which indicated the loss of the caffeic acid moiety from the $[M-H]^-$ ion as well as the typical fragment of caffeic acid at *m*/*z* 179.

Furthermore, a compound with an $[M-H]^-$ ion at *m*/*z* 353 (**F4**) was detected and identified as chlorogenic acid (5-*O*-caffeoylquinic acid) by comparison of retention time, UV and mass spectra with those of an authentic reference substance. Similarly, compound **F7** (*m*/*z* 163) was identified as p-coumaric acid. Two compounds (**F5**) exhibiting an $[M-H]^-$ ion at *m*/*z* 325, were detected after 38.2 and 39.7 min indicating the occurrence of different isomeric structures and were identified as coumaric acid-hexosides based on the fragments at *m*/*z* 163 (loss of a hexose moiety) and the typical fragment of coumaric acid at *m*/*z* 119.

Finally, another hydroxycinnamic acid (**F6**) with an $[M-H]^-$ ion at *m*/*z* 355 was detected. Its fragmentation revealed the formation of a prominent aglycone fragment at *m*/*z* 193 also showing demethylated and decarboxylated product ions. **F6** was therefore assigned to a ferulic acid derivative containing a hexose moiety.

3.2.6. Dihydroflavonols

Moreover, a minor component (**G1**) was detected in all juices. Fragmentation of the $[M-H]^-$ ion at *m*/*z* 449 was characterised by the loss of a hexose moiety (162 Da) and further dehydration (loss of 18 Da), resulting in product ions at *m*/*z* 287 and 269. Two isomeric compounds with similar fragmentation patterns were observed (retention times of 41.9 and 44.4 min), and compound **G1** was identified as dihydrokaempferol-hexoside since the fragmentation pattern and the UV/Vis absorption spectrum were consistent with previous reports (Regos, Urbanella, & Treutter, 2009). To our knowledge this compound has not been reported in *P. granatum* L. so far.

3.3. Quantification of individual phenolic compounds in pomegranate peel, mesocarp and juices

Despite a great number of studies, comparison of the phenolic contents with literature data is still aggravated due to differing analytical methodologies and because the contents may considerably vary with the variety and maturity stage of pomegranates (Hajimahmoodi et al., 2008; Mousavinejad, Emam-Djomeh, Rezaei, & Khodaparast, 2009). Furthermore, in previous studies only the predominant phenolic compounds were determined not considering minor components.

Antioxidant capacity as deduced from the TEAC assay and total phenolics measured applying the Folin–Ciocalteu assay exhibited highest values for the mesocarp, followed by pomegranate peels (Table 3). For all samples the amounts of total phenolics as determined by HPLC showed good agreement with the data obtained from the Folin–Ciocalteu assay ($R^2 = 0.9991$). TEAC and FRAP values were also highly correlated ($R^2 = 0.9995$). The FRAP assay revealed superior antioxidant capacity of the mesocarp; however, the differences between mesocarp and peel extracts were insignificant. Among the juices, variant 2 showed highest values in all three test assays compared to variants 1 and 3. This can be seen from the results of the TEAC assay revealing antioxidant activities of 76.4 mmol/L in juice 2 compared to 24.5 and 21.7 mmol/L in juices 1 and 3, respectively. Accordingly, the antioxidant activity of 42 mmol/L trolox equivalents of a commercial juice from the entire fruit as reported by Seeram et al. (2008) is within this range.

Table 4
Antioxidant activity of isolated phenolics measured by the TEAC assay.[a]

Compound (1 mmol/L)	Trolox equivalents (mmol/L)
Punicalagin	6.3 ± 0.3
Ellagic acid	0.4 ± 0.1
Gallic acid	0.9 ± 0.0
Protocatechuic acid	0.4 ± 0.0
p-Coumaric acid	0.5 ± 0.0

[a] Values expressed as means of duplicate determinations ± standard error.

Çam, Hişil, and Durmaz (2009) determined TEAC values of different pomegranate juices obtained by pressing the seeds of cultivars widely grown in Turkey. The TEAC values ranged from 9 to 17 mmol/L and, thus come close to that of juice variant 3, which was also pressed from arils and seeds.

In a further study, similar TEAC values (12–14 mmol/L) were determined in juices obtained from arils, whereas commercial juices, obtained by pressing the entire fruit generally showed higher values ranging from 18 to 20 mmol/L (Gil et al., 2000).

Pomegranate has been propagated as a polyphenol-rich food with health beneficial effects due to its high antioxidative capacity, thus is being commonly referred as "superfruit". Seeram et al. (2008) compared the antioxidative capacity of different polyphenol-rich beverages, such as different kinds of juices and tea as well as red wine. Among these beverages, pomegranate juice was found to exhibit the highest TEAC value (42 mmol/L), followed by red wine (19 mmol/L), Concord grape juice (17 mmol/L) and blueberry juice (15 mmol/L). Açai and cranberry fruits, also belonging to the "superfruits", displayed inferior antioxidant capacities of 13 and 9 mmol/L trolox equivalents in their juices. However, the *in vitro* antioxidative capacity may not correlate with antioxidant effects in humans, since the bioavailability and bioconversion are not taken into consideration. Nevertheless, in several studies pomegranate juice has been demonstrated to exert anti-inflammatory effects in humans (Aviram et al., 2004; Pantuck et al., 2006; Rosenblat, Hayek, & Aviram, 2006).

As can be seen from Table 5, ellagitannins were the predominant phenolic compounds in pomegranate peels, mesocarp, arils and juices, with punicalagin (HHDP-gallagyl-hexoside) showing the highest amount, except for juices derived form isolated arils (variant 3). Among the ellagitannins punicalagin (**D2**) and bis-HHDP-hexoside (peduncalagin, **C7**) were quantified with standard compounds which were isolated by preparative HPLC (Section 2.3). Compound purity was 93% and 94% for punicalagin and peduncalagin, respectively, as deduced from the ratio of peak areas of the isolated compounds and total peak area at 280 nm. Both compounds were also used for the quantification of structurally related compounds (Section 2.6). The greatest diversity of ellagitannins (21 components) was found in the peel, followed by the mesocarp (19 components), juice variant 1 (17 components), juice variant 2 (16 components) and juice variant 3, which only comprised 11 different ellagitannins. Also the total amounts of ellagitannins in different parts of pomegranate and juices varied significantly. A more complex ellagitannin profile was associated with deviated ellagitannin contents.

In addition to punicalagin (**D2**, 10.5 g/kg), very high levels of galloyl-HHDP-hexoside (**C6**, 3.9 g/kg), granatin B (**C17**, 5.9 g/kg), digalloyl-HHDP-gluconic acid (**C21**, 3.8 g/kg) and bis-HHDP-hexoside (**C7**, 3.5 g/kg) were found in pomegranate peels. In contrast, minor amounts of ellagic acid and its derivatives (**C1**–**C5**, $\leqslant$1 g/kg) were detected in the peel and mesocarp. Similar results were also observed with the juices extracted by applying elevated pressures. Bis-HHDP-hexoside (**C7**) and galloyl-HHDP-hexoside (**C6**) were the predominant ellagitannins in juice variants 1 and 2. In addition, compounds **C17**–**21** and **D2** had significant amounts in variant 2, indicating that higher pressures result in a more complex ellagitannin profile. Accordingly, only low amounts of ellagitannins were detected in juice variant 3, which was prepared from arils. In this extraordinary case, the ellagitannin fraction was dominated by

Table 5
Contents of individual phenolic compounds in juices, peel and mesocarp of pomegranate.[a,b,c,d]

	Compound	Juice variant 1 (mg/L)	2 (mg/L)	3 (mg/L)	Peel (mg/kg DM)	Mesocarp (mg/kg DM)
A1	Del-3,5-diglc	10.8 ± 0.1	0.4 ± 0.0	205.4 ± 23.2	10.8 ± 0.3	–
A2	Cya-3,5-digc	150.3 ± 1.0	95.4 ± 2.3	272.1 ± 15.3	157.8 ± 7.1	–
A3	Pel-3,5-diglc	16.8 ± 0.3	13.7 ± 0.1	26.7 ± 1.0	145.8 ± 15.5	–
A4	Del-3-glc	1.6 ± 0.0	1.0 ± 0.0	16.8 ± 3.4	13.3 ± 0.5	–
A5	Pent-hex	1.6 ± 0.2	1.1 ± 0.0	3.0 ± 0.2	0.0 ± 0.0	–
A6	Cya-3-glc	12.6 ± 0.2	9.1 ± 0.0	27.9 ± 4.7	41.2 ± 0.8	–
A7	Cya-3-rutin	1.2 ± 0.1	0.8 ± 0.0	0.0 ± 0.0	18.4 ± 0.3	–
A8	Pel-3-glc	2.9 ± 0.0	2.4 ± 0.0	4.9 ± 0.7	56.7 ± 1.8	–
A9	Cya-3-hex	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	1.7 ± 0.5	–
A10	Cya-3-pent	0.3 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	1.4 ± 0.0	–
Total anthocyanins		**198.3** ± 0.8b	**124.2** ± 2.2b	**557.7** ± 48.3a	**447.1** ± 11.3	–
B1	Galloylhex	11.4 ± 1.5	–	2.7 ± 0.2	–	–
B2	Digalloylhex	9.3 ± 0.1	3.4 ± 0.0	nq	12.2 ± 0.7	29.6 ± 0.5
	Total gallotannins	**9.1** ± 0.5a	**1.2** ± 0.0b	**1.4** ± 0.1b	**4.3** ± 0.2x	**10.4** ± 0.2y
C1	Ellagic acid	4.6 ± 0.2	7.2 ± 0.5	2.1 ± 0.0	637.7 ± 32.8	234.2 ± 13.0
C2	HHDP-hex	4.3 ± 0.4	–	5.7 ± 0.0	507.4 ± 13.5	nq
C3	Ellagic acid-hex	4.2 ± 0.2	9.0 ± 0.3	1.7 ± 0.1	956.8 ± 1.3	466.7 ± 31.5
C4	Ellagic acid-pent	1.4 ± 0.1	0.8 ± 0.0	2.9 ± 0.2	618.2 ± 33.2	30.1 ± 1.0
C5	Ellagic acid-deoxyhex	3.2 ± 0.2	1.1 ± 0.1	3.9 ± 0.1	–	42.5 ± 1.6
C6	Galloyl-HHDP-hex	62.7 ± 2.1	96.3 ± 3.5	nq	3994.8 ± 157.5	587.5 ± 171.6
C7	bis-HHDP-hex	110.6 ± 14.3	329.6 ± 71.9	30.0 ± 1.7	3498.8 ± 156.7	1954.6 ± 210.3
C8	Digalloyl-HHDP-hex	70.7 ± 2.2	64.5 ± 10.1	39.2 ± 1.9	1829.4 ± 31.2	2461.5 ± 243.7
C9	Galloyl-bis HHDP-hex	–	–	–	2086.5 ± 138.8	243.2 ± 4.1
C10	Ellagic acid der	0.4 ± 0.0	–	–	65.3 ± 1.5	11.9 ± 2.4
C11	Ellagic acid der	0.4 ± 0.0	–	0.6 ± 0.1	–	–
C12	Ellagic acid dihex	1.3 ± 0.0	–	–	–	–
C13	Valoneic acid bilactone	nq	nq	–	439.9 ± 23.3	nq
C14	Ellagic acid der	–	–	0.20 ± 0.02	–	–
C15	Lagerstannin B	–	–	–	2118.0 ± 71.1	nq
C16	Lagerstannin B der	–	–	–	1666.5 ± 72.3	–
C17	Granatin B	–	304.3 ± 8.6	–	5868.7 ± 351.3	2970.9 ± 41.6
C18	Castalagin der	–	234.1 ± 0.7	–	2936.9 ± 547.3	1081.3 ± 240.1
C19	Ellagic acid der	43.8 ± 2.2	145.2 ± 6.6	–	352.0 ± 10.0	2288.6 ± 534.8
C20	Galloyl-HHDP-gluc acid	24.7 ± 2.3	84.2 ± 4.3	–	1912.3 ± 308.5	5692.4 ± 673.1
C21	Digalloyl-HHDP-gluc acid	5.6 ± 0.7	185.7 ± 6.0	–	3779.1 ± 147.5	2209.2.6 ± 58.3
C22	Brevifolin carboxylic acid	0.5 ± 0.0	2.7 ± 0.3	–	32.7 ± 2.7	32.9 ± 2.4
	Ellagitannins[c]	340.1 ± 15.2b	1492.5 ± 28.8a	86.4 ± 3.1c	33305.6 ± 76.8x	20280.6 ± 3602.3x
D1	Gallagyl-hex	9.0 ± 0.2	4.0 ± 0.4	–	130.0 ± 3.6	–
D2	HHDP-gallagyl-hex	54.0 ± 1.2	564.5 ± 18.9	4.1 ± 0.2	10543.4 ± 468.0	20314.8 ± 701.0
	Gallagyl esters	63.0 ± 1.0b	578.5 ± 18.5a	4,1 ± 0.2c	10673.4 ± 471.6x	20314.8 ± 831.9.0y
	Total ellagitannins	**403.1** ± 16.2b	**2071.0** ± 47.3a	**90.4** ± 3.4c	**43979.0** ± 394.8x	**40595.4** ± 4434.2x
Total hydrolysable tannins		**423.8** ± 15.3b	**2074.4** ± 47.3a	**93.2** ± 3.6c	**43991.2** ± 395.5x	**40625.1** ± 4434.7x
E1	Gallic acid	2.1 ± 0.1	1.1 ± 0.0	1.8 ± 0.1	270.4 ± 18.5	nq
E2	Protocatechuic acid	0.8 ± 0.0	–	nq	–	–
E3	Protocatechuic acid-der	2.2 ± 0.0	–	7.3 ± 0.3	–	–
E4	Vanillic acid 4-glc	1.8 ± 0.1	nq	1.5 ± 0.2	–	–
Total hydroxybenzoic acids		**6.9** ± 0.0a	**1.1** ± 0.0b	**10.6** ± 0.3a	**270.4** ± 18.5	–
F1	Caffeic acid hex	5.2 ± 0.4	–	3.4 ± 0.0	–	–
F2	Caffeic acid hex der	6.8 ± 0.8	–	10.8 ± 1.3	–	–
F3	Caffeic acid der	6.1 ± 0.2	nq	7.6 ± 0.4	–	–
F4	5-*O*-Caffeoylquinic acid	1.6 ± 0.1	–	–	–	–
F5	Coumaric acid hex	1.1 ± 0.0	–	2.9 ± 0.2	–	–
F6	Ferulic acid hex	–	–	3.6 ± 0.1	–	–
F7	Coumaric acid	0.6 ± 0.0	–	nq	–	–
Total hydroxycinnamic acids		**21.4** ± 0.9b	–	**28.3** ± 1.4a	–	–
G1	Dihydrokaempferol-hex	1.4 ± 0.3	nq	nq	–	–
Total phenolics[d]		**453.6** ± 18.4b	**2075.4** ± 47.3a	**132.1** ± 5.3c	**44261.5** ± 414.0x	**40625.1** ± 4434.7x

[a] Abbreviations: cya, cyanidin; del, delphinidin; deoxyhex, deoxyhexoside; der, derivative; glc, glucoside; gluc, gluconic; hex, hexoside; nq, not quantifiable; pel, pelargonidin; pent, pentoside; rt, retention time; rut, rutinoside; – not detected.
[b] Values expressed as means of duplicate determinations ± standard error. Significant differences between values in the same line are indicated by different letters a–c and x/y ($P < 0.05$).
[c] Without gallagyl esters.
[d] Without anthocyanins.

bis-HHDP-hexoside (**C7**) and digalloyl-HHDP-hexoside (**C8**), whereas galloyl-bis-HHDP-hexoside (**C9**), ellagic acid dihexoside (**C12**), valoneic acid bilactone (**C13**), granatin B (**C17**), both ellagitannins with a gluconic acid core (**C20** and **C9**), the gallagyl-hexoside

(**D1**), as well as the hydroxycinnamic acid **F4** and some derivatives such as the compounds **C10**, **C18** and **C19** are lacking in this juice. The compounds **B2**, **C6**, **E2** and **F7** were not quantifiable.

The dried mesocarp exhibited the maximum antioxidative potential and the highest amounts of total phenolics as deduced from the Folin–Ciocalteu assay and the quantitative determination of gallagyl esters, but lower amounts of simple ellagitannins compared to the peel. Thus, it can be concluded, that the antioxidative capacity is strongly correlated with the amounts of gallagyl esters, especially punicalagin. Obviously, even considerable amounts of simple ellagitannins do not markedly contribute to the antioxidative properties. These assumptions were verified with standard components, which were analysed applying the TEAC assay (Table 4), with 1 mmol/L punicalagin solutions showing a predominant antioxidative capacity as deduced from a TEAC value of 6.3 mmol/L, followed by gallic acid with only 0.9 mmol/L trolox equivalents. Ellagic acid, protocatechuic acid and coumaric acid showed only minor antioxidative capacities of 0.4, 0.4 and 0.5 mmol/L trolox equivalents, respectively. In another study, an even lower TEAC value for gallic acid was determined with 0.2 mmol/L (Tafulo, Queirós, Delerue-Mato, & Sale, 2010).

Even though the amounts of ellagitannins and total hydrolysable tannins were comparable in dried peels (42% DM) and dried mesocarp (20% DM) revealing contents of 44 and 41 g/kg, respectively, the proportion of punicalagin based on the total ellagitannin contents was double in dried mesocarp (20.3 g/kg) compared to dried peels (10.5 g/kg). However, on a fresh weight basis, the punicalagin proportions of 4.5 and 4.2 g/kg in the peels and mesocarp, respectively, were comparable, which is due to the differences in the dry matter content. This was also observed for the other phenolic compounds. Accordingly, total phenolic amounts of the fresh peels were higher than of the fresh mesocarp.

Much higher punicalagin contents ranging from 40 to 98 g/kg were determined in another study in pomegranate peels of 14 varieties (Lu et al., 2008), demonstrating a large variability of polyphenol contents. In the present study, punicalagin contents of pomegranate juices ranged from 4 mg/L (variant 3) to 54 mg/L (variant 1) and 565 mg/L (variant 2). The highest value was obtained by applying maximum pressure. Gil et al. (2000) reported punicalagin contents of 20–1350 mg/L in pomegranate juices, with the lowest contents being also found in juices solely pressed from arils. Furthermore, total gallagyl ester contents of 70–1800 mg/L were determined in this study (Gil et al., 2000). Compared to these data, the total amounts of gallagyl esters in the juices were lower ranging from 4 to 579 mg/L and, accordingly, in the same dimension as the punicalagin contents.

Only a minor part of pomegranate phenolics was constituted of hydroxybenzoic acids, hydroxycinammic acids and the dihydrokaempferol-hexoside. Among these, only gallic acid was detected in the peel, mesocarp and all juice variants. All other phenolics of these compound classes were exclusively detected in the juice obtained by applying minimum pressure (variant 1) and in the juice obtained from the arils (variant 3). The latter juice was characterised by the highest amounts of hydroxycinnamic acids. Such low amounts as compared to the hydrolysable tannins, could not be detected in the juice obtained by applying maximum pressure, because the very high contents of the latter compounds made quantification of these minor compounds in juice variant 3 impossible.

In contrast to its low phenolic contents, juice variant 3 was extremely rich in anthocyanins (558 mg/L). Cyanidin-3,5-diglucoside was the predominant compound among the pigments, followed by delphinidin-3,5-diglucoside. In contrast, pelargonidin-3,5-diglucoside was the second most important anthocyanin in the peels. This also conformed to juice variants 1 and 2, with variant 1 showing with 198 mg/L slightly higher total anthocyanin contents than variant 2 with 124 mg/L. However, differences between both juices were insignificant. In further studies, comparative total anthocyanin amounts of 172 and 387 mg/L were determined in juices from arils (Gil et al., 2000). Interestingly, the peels constitute a rich source of anthocyanins, which are not exploited when isolated arils were dejuiced.

This study clearly demonstrates LC–MS^n to be a powerful tool for the characterisation of anthocyanins and non-anthocyanin phenolic compounds in pomegranate peels, mesocarp and juice. More than 35 phenolic compounds were characterised and quantified in pomegranate, and after preparative isolation pedunculagin was used for the first time as reference compound. The phenolic profiles and contents of pomegranate juices were significantly affected by the processing method. Thus, polyphenol profiles of pomegranate juices may be used to distinguish between aril based products and those including peel and mesocarp. These findings may help to further elucidate the health-promoting potential of pomegranates and products derived therefrom, such as juices and medical preparations, and to standardise such products based on the contents of their active principles.

The popular application of pomegranate juices in the adjuvant treatment of prostate cancer may be partly supported by the first finding of brevifolin carboxylic acid in the fruit, since strong cytotoxic activity against human tumour cell lines has been reported for this compound by Lee and Yang (1994). Furthermore, brevifolin carboxylic acid was also found to have β-glucuronidase inhibitory action, which was suggested as potential hepaprotective agents (Yoshi & Priya, 2007). This compound was also proposed for the prevention and treatment of retrovirus related disease, such as the human immunodeficiency virus (HIV) (Wagner & Notka, 2004).

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