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Original Research Article

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

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ABSTRACT

"Wonderful" pomegranate (*Punica granatum*) juice, obtained by pressure extraction of the whole fruit, was analyzed for its content in anthocyanin and non-anthocyanin phenolic components using high-performance liquid chromatography with diode array detection and tandem mass spectrometry analysis with positive and negative electrospray ionization (HPLC-DAD-ESI$^{+/-}$/MS^n) powered by an ion trap. High-throughput identification capacity from the ion trap featuring different MS^n experiments (reaching up to MS^4 level) led to detection of a total of 151 phenolics, 65 anthocyanin, anthocyanin–flavanol and flavanol–anthocyanin adducts, 25 of them reported for the first time in pomegranate juice, including some unusual cyanidin and pelargonidin trihexosides not previously described in natural extracts. Similarly, a total of 86 non-anthocyanin phenolic components were also identified, 39 of them reported for the first time in this juice.

1. Introduction

In recent years, consumers have witnessed the apparition and growth of a series of foodstuffs that make claims about their antioxidant properties and goodness for human health. Such products (popularly known as "ANTIOX") are intended for preventing disorders caused by aging, pollutants, carcinogenic agents and stress, among others, and are normally based on a high content in antioxidants (Kaur and Kapoor, 2001). They are usually commercialized either as concentrated tablets from extracts of natural products or as beverages from different fruit juices, provided that such beverages exhibit an attractive color. Hence, most of these commercial "ANTIOX" beverages are based on juices that are rich in anthocyanins, such as red grapes, berries and pomegranate, among others, which in addition to their strong antiradical activity also exhibit a nice color. It is noteworthy that pomegranate juice has become so popular in some developed countries that many consumers believe it to be a preferred food to be included in everyday nutrition. As a consequence, pomegranate fruit and juice have been catapulted into a prominent position in international commerce (Zhang et al., 2009).

Pomegranate juice is very rich in phenolic components, including anthocyanins which are the responsible of its nice red color. Majority of the bibliographic information reports only the presence of six major anthocyanins in pomegranate juice, namely the 3-mono- and 3,5-diglucoside derivatives of the anthocyanidins delphinidin, cyanidin and pelargonidin (Du et al., 1975; Gil et al., 2000; Alighourchi et al., 2008; Elfalleh et al., 2011). However, there have recently been reported a quite large number of colored anthocyanins-flavanol (Sentandreu et al., 2010) and flavanol–anthocyanin (Sentandreu et al., 2012) adducts, but at very low concentration. With respect to the presence of phenolic components others than anthocyanins (Gil et al., 2000; Madrigal-Carballo et al., 2009; Borges et al., 2010), almost all the consulted bibliography reports a number between 5 and 15, mainly ellagitannins and gallotannins, excepting a more recent study (Fischer et al., 2011) which reported the presence of about 55 of these metabolites. It must be noted that natural extracts use to be complex matrices with intricate metabolite composition, thus the use of ion traps (normally reaching a mass fragmentation analysis up to MS^3 level) has been claimed for molecular structure elucidation (Fountain, 2002; Kantharaj et al., 2003).

The aim of this work was to thoroughly study the phenolic composition of pomegranate juice, including both anthocyanin and non-anthocyanin compounds, by using the high-performance liquid chromatography with diode array detection and tandem

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mass spectrometry analysis with positive and negative electrospray ionization (HPLC-DAD-ESI$^{+/-}$/MS^n) methodology with an ion trap mass analyzer.

2. Materials and methods

2.1. Reagents and standards

Water, acetonitrile, tetrahydrofuran (THF) and formic acid of analytical grade were from Scharlab (Scharlab S.L., Barcelona, Spain). Nylon filters (0.45 μm) were from Teknokroma (Teknokroma Ltd., Barcelona, Spain).

2.2. Pomegranate juice

Pomegranate fruits, cultivar "Wonderful", were grown and imported from CA, USA. Pomegranate juice was obtained by pressure extraction of the whole fruit (50 units of fruit which weighted 25 kg), previously cut into halves, using a pressure extractor Europ (Vapfluid, Sant Boi de Llobregat, Barcelona, Spain) working at an air pressure of 6 kg/cm^2. Juice was sieved in a paddle finisher (Ø 0.4 mm, Luzzysa, El Puig, Spain) and yield of juice was 42% (w/w), having the following characteristics: °Brix, 17.2 (determined using a digital refractometer Pal-1; Atago Co. Ltd., Tokyo, Japan); pH 3.3 (determined using a Crison GLP 21 pH-meter; Crison Inst. S.A., Barcelona, Spain); acidity index, 1.07 (assessed by titration with 0.1 N NaOH and expressed as percentage of citric acid); dry matter, 12.7% (determined by oven dying at 70 °C until constant weight); and maturity index, 16.1. Immediately after extraction, separate aliquots (20 mL) of the juice were poured into vials, which were hermetically sealed and stored at −30 °C until analysis (two months as a maximum).

2.3. Sample preparation

An aliquot of the juice was defrosted at room temperature and darkness in a water bath and then centrifuged at 5000 rpm during 5 min. The supernatant was filtered through a 0.45 μm nylon filter and then injected into the platform of analysis.

2.4. HPLC-DAD-ESI/MS^n analysis

Chromatographic separation was performed using a Thermo Surveyor Plus HPLC (Thermo Scientific, San Jose, CA, USA), equipped with a quaternary pump, vacuum degasser and temperature-controlled autosampler. The column used was a 250 mm × 2.1 mm i.d., 3 μm, YMC C-18 pack-*pro* (YMC Europe GmbH, Dinslaken, Germany). The chromatographic conditions were: injection volume, 10 or 5 μL for the analysis of anthocyanins or non-anthocyanin phenols, respectively; flow rate, 0.2 mL/min; oven temperature, 24 °C; autosampler temperature, 10 °C; solvent A, water/THF/formic acid (97.5:2.0:0.5, v/v/v); solvent B, acetonitrile/THF/formic acid (97.5:2.0:0.5, v/v/v); gradient, initial 0% B, linear 0–6% B in 5 min, linear 6–18% B in 25 min, linear 18–80% B in 20 min, purging with 100% B during 10 min and re-equilibration of the column during 30 min; detection wavelengths, λ = 280, 320 and 520 nm.

MS analysis and fragmentation experiments were performed on a ThermoFinningan LCQ Advantage (Thermo Scientific, San Jose, CA, USA) ion trap mass spectrometer, equipped with an ESI source. The HPLC-DAD/MS^n platform was controlled and the resulting data were analyzed by using the software Xcalibur v. 2.06, loaded into a PC computer. Separate injections were run for analysis of the sample in both positive and negative electrospray ionization (ESI) modes.

2.4.1. Identification of anthocyanins, flavanol–anthocyanin and anthocyanin–flavanol adducts

The mass spectrometer was operated in the ESI positive ion mode under the common following conditions: source voltage, 3.5 kV; capillary voltage, 9 V; capillary temperature, 300 °C; sheath gas flow, 50 (arbitrary units); sweep gas flow, 20 (arbitrary units); full max ion time, 300 ms; and full micro scans, 3.

The ion trap mass analyzer used in this work is quite aged and so it hardly allows more than four scan events per injected sample. Therefore, it was necessary to run many separate injections to maximize its sensitivity and selectivity, so avoiding an excessive number of scan events per injected sample, and each run was subjected to a specific set of a few MS^n experiments. Moreover, the MS^n experiments were of two types, namely, general data dependent scan analyses and specific non-data dependent scan analyses. The first general type gives information about the major components of the juice but normally neglects the minor ones. The second type was planned to gain information about these minor components as well as to clarify dubious ion fragmentation sequences from the first general type.

2.4.1.1. Data dependent scan analyses. Data dependent scan analyses were carried out with the following general conditions: collision energy, 35% (arbitrary units); reject mass-to-charge ratio (*m*/*z*) width, 1.00; repeat count, 2; repeat duration, 0.5 min; exclusion size list, 25; exclusion duration, 1.00 min; exclusion mass width, 3.00.

General MS^3 analysis. This analysis included three scan events: *scan event 1*, full MS; *scan event 2*, MS^2 of the most intense ions from *scan event 1*; and *scan event 3*, MS^3 of the most intense ions from *scan event 2*. The scanned *m*/*z* range was 260–2000.

General MS^2 analysis for the detection of anthocyanin monoglycosides. This analysis included two scan events: *scan event 1*, full MS; and *scan event 2*, MS^2 of the ions populated in the target list: *m*/*z* 403, 417, 419, 433, 435, 449 and 465. The scanned mass range was *m*/*z* 260–500.

General MS^3 analysis for the detection of anthocyanin diglycosides. This analysis included three scan events: *scan event 1*, full MS; *scan event 2*, MS^2 of the ions populated in the target list: *m*/*z* 565, 579, 581, 595, 597, 611 and 627; and *scan event 3*, MS^3 of the most intense ion from *scan event 2*. The scanned mass range was *m*/*z* 260–700.

Neutral loss triple play analysis. This analysis was carried out to determine the presence of major anthocyanin glycosides and included three scan events: *scan event 1*, full MS; *scan event 2*, MS^2 of the four most intense ions from *scan event 1* which undergo any of the following neutral losses: *m*/*z* 132, 146, 162, 294, 308, 324 or 486; and *scan event 3*, MS^3 of the most intense ion generated in *scan event 2*. The scanned mass range was *m*/*z* 260–2000.

2.4.1.2. Non-data dependent scan analyses. Specific MS^3 analysis for the detection of rutinoside derivatives of anthocyanidins. Three separate injections (I–III) were run for the detection of rutinoside derivatives of anthocyanins, each one including three specific scan events: *scan event 1*, common full MS; *scan event 2*, specific MS^2 of the ion at *m*/*z* 579 (I), 595 (II) and 611 (III); and *scan event 3*, specific MS^3 of the ion at *m*/*z* 433 (I), 449 (II) and 465 (III). The scanned mass range was *m*/*z* 260–650.

Specific MS^4 analysis for the detection of dihexoside derivatives of anthocyanin–*flavanol and flavanol–anthocyanin adducts.* Five separate injections (I–V) were run, each one including four specific scan events: scan event 1, common full MS; scan event 2, specific MS^2 of the ion at *m*/*z* 867 (I), 883 (II), 899 (III), 915 (IV) and 931 (V); scan event 3, specific MS^3 of the ion at *m*/*z* 705 (I), 721 (II), 737 (III), 753 (IV) and 769 (V); and scan event 4, specific MS^4 of the ion at *m*/*z*

543 (I), 559 (II), 575 (III), 591 (IV) and 607 (V). The scanned mass range was *m/z* 260–1000.

Specific MS³ analysis for the detection of monohexoside derivatives of anthocyanin–*flavanol and flavanol–anthocyanin adducts.* Five separate injections (I–V) were run, each one including three specific scan events: scan event 1, common full MS; scan event 2, specific MS² of the ion at *m/z* 705 (I), 721 (II), 737 (III), 753 (IV) and 769 (V); and scan event 3, specific MS³ of the ion at *m/z* 543 (I), 559 (II), 575 (III), 591 (IV) and 607 (V). The scanned mass range was *m/z* 260–800.

2.4.2. Identification of the non-anthocyanin phenolic components

The mass spectrometer was operated in the ESI negative ion mode under the following conditions: source voltage, 4.0 kV; capillary voltage, −18 V; capillary temperature, 300 °C; sheath gas flow, 50 (arbitrary units); sweep gas flow, 20 (arbitrary units); full max ion time, 300 ms, and full micro scans, 3. Again and to maximize the sensitivity and selectivity of the ion trap mass analyzer, several separate injections of the sample were run. However, in this case only data dependent scan experiments were carried out by using the same collision energy and dynamic exclusion settings previously described for the detection of anthocyanins.

General MS³ analysis. This general MS³ analysis was all in all similar to that described for anthocyanins, excepting that the scanned mass range was *m/z* 100–2000.

Neutral loss triple play analysis for detecting the loss of carboxylic groups. This analysis included three scan events: *scan event 1*, full MS; scan event 2, MS² of the four most intense ions from *scan event 1* which undergo a neutral loss of 44 amu; and *scan event 3*, MS³ of the most intense ion generated from *scan event 2*. The scanned mass range was *m/z* 100–2000.

Neutral loss triple play analysis for detecting the loss of functional groups. In a similar way to the above section, five separate injections (I–V) were run for detecting the loss of different functional groups. In this case, however, the specified losses of neutral fragments were: (I) *m/z* 132, 146, 162, 294, 308, 324 and 486 for sugars; (II) *m/z* 152 and 170 for gallic or galloyl derivates; (III) *m/z* 180 and 302 for hexoside derivatives of hexahydroxydiphenic acid (HHDP), quercetin and ellagic acid; (IV) *m/z* 276 and 602 for 3,4,8,9,10-pentahydroxy-dibenzo[b,d]pyran-6-one and gallagyl derivatives; and (V) *m/z* 46 for formic acid adducts. In all cases, the scanned mass range was *m/z* 100–2000.

3. Results and discussion

3.1. Anthocyanins profiling

Fig. 1A shows the HPLC chromatogram of pomegranate juice acquired at λ = 520 nm, wavelength at which practically only the anthocyanins and anthocyanins adducts are detected. In this chromatogram, the six major peaks (numbered as in Table 1) correspond to the well-known 3-glucoside and 3,5-diglucoside derivatives of the anthocyanidins delphinidin, cyanidin and pelargonidin. A zooming of this chromatogram, as shown in Fig. 1B, reveals the presence of a rather large number of other components that also absorb at this wavelength, thus suggesting that presence of additional anthocyanidin derivatives in pomegranate juice, but at much lower concentrations. Table 1 lists the 65 anthocyanins, flavanol-anthocyanin and anthocyanin–flavanol adducts detected in this work, as well as their chromatographic retention times and MS^n fragmentation patterns.

3.1.1. Colored flavanol-anthocyanin and anthocyanin–flavanol adducts

A total of thirty 3-hexoside and 3,5-dihexoside derivatives of flavanol-anthocyanin and anthocyanin–flavanol adducts were

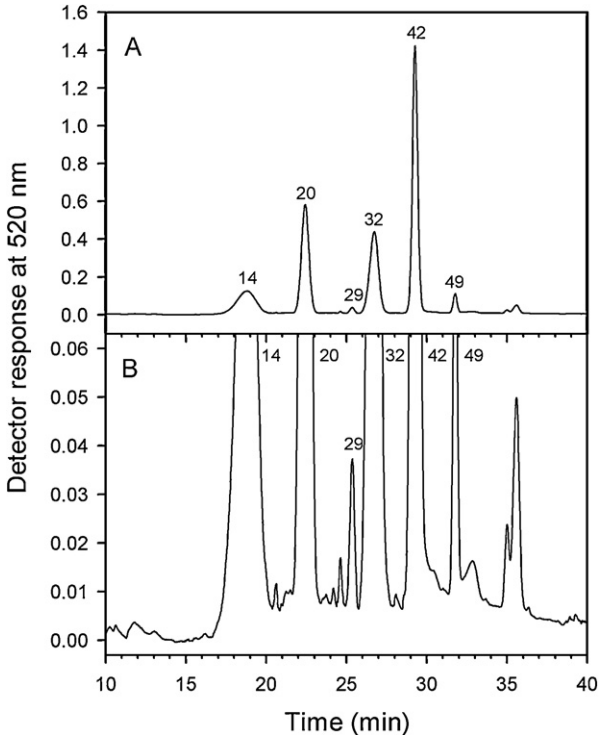

Fig. 1. HPLC chromatogram of pomegranate juice acquired at λ = 520 nm. (A) Normalized standard chromatogram showing the major anthocyanin components: delphinidin-3,5-diglucoside (peak 14), cyanidin-3,5-diglucoside (peak 20), pelargonidin-3,5-diglucoside (peak 29), delphinidin-3-glucoside (peak 32), cyanidin-3-glucoside (peak 42), and pelargonidin-3-glucoside (peak 49). (B) Zoomed chromatogram showing additional minor anthocyanin components.

detected in this work. This number is smaller than the reported one in bibliography for the same variety (“Wonderful”) of pomegranate (Sentandreu et al., 2010, 2012), but this discrepancy could be due to the different ripening state of the analyzed fruits and/or to their different origin, California (this work) and Peru. In any case, these components were present at very low concentration.

3.1.2. Tri-substituted anthocyanidin derivatives

As shown in Table 1, peaks 3 and 8 exhibit a molecular ion $[M]^+$ at *m/z* 789. Its MS² yields fragments at *m/z* 627 ($[M]^+$-162), 465 ($[M]^+$-324) and 303 ($[M]^+$-486), thus indicating a tri-hexoside derivative of delphinidin. This fragmentation sequence, without additional information, is compatible with the structure of delphinidin-3-diglucoside-5-glucoside (Olsen et al., 2010), but in this case the MS³ of the daughter ion at *m/z* 627 would yield a lonely ion at *m/z* 303 ($[M]^+$-162-324). In our case, however, the MS³ of the daughter ion at *m/z* 627 yielded ions at *m/z* 465 ($[M]^+$-162-162) and 303 ($[M]^+$-162-162-162), thus indicating a tri-hexoside derivative of delphinidin, but with each hexoside located at a different position within the aglycone structure. To our best knowledge, no one free tri-substituted hexoside derivative of delphinidin has been reported in bibliography from a natural source. Nevertheless, Yoshitama et al. (1975) identified delphinidin-3,7,3′-trihexoside from the deacylation of cineranin, a pigment from the blue flowers of Cineraria (*Senecio cruentus* DC.), whereas Fukuchi-Mizutani et al. (2003) demonstrated that the activity of the specific anthocyanin-3′-O-glucosyltransferases, from Gentian (*Gentiana triflora*) petals, on delphinidin-3,5-diglucoside yields delphinidin-3,5,3′-triglucoside.

Similarly to peaks 3 and 8, peaks 6 and 11 exhibit a molecular ion $[M]^+$ at *m/z* 773 and its MS² yields fragments at *m/z* 611

Table 1
Anthocyanins detected in pomegranate juice.

Rt (min)	Peak no.	$[M]^+$ (*m*/*z*)	MS^2	MS^3	MS^4	Identification[a]
5.58	1	**931**	**769**, 607	**607**	589, 481, 439, 345, 303	*(epi)gcat-dpd-3,5-dihexoside*
6.88	2	**931**	**769**, 607	**607**	589, 481, 439, 345, 303	*(epi)gcat-dpd-3,5-dihexoside*
7.67	3	**789**	**627**, 465, 303	465, 303		Dpd-trihexoside
7.99	4	**815**	**753**, 591	**591**	573, 465, 423, 329, 287	*(epi)gcat-cyd-3,5-dihexoside*
8.23	5	**627**	**465**, 303	303		Dpd-3,5-dihexoside
8.82	6	**773**	**611**, 449, 287	449, 287		Cyd-trihexoside
10.65	7	**769**	**607**	589, 481, 439, 345, 303		*(epi)gcat-dpd-3-hexoside*
12.17	8	**789**	**627**, 465, 303	465, 303		Dpd-trihexoside
13.82	9	**627**	**465**, 303	303		Dpd-3,5-dihexoside
14.07	10	**915**	**753**, 591	**591**	573, 465, 439, 345, 303	*(epi)cat-dpd-3,5-dihexose*
15.76	11	**773**	**611**, 449, 287	449, 287		Cyd-trihexoside
16.56	12	**753**	**591**	573, 465, 423, 329, 287		*(epi)gcat-cyd-3-hexoside*
17.97	13	**931**	**769**, 607	**607**	589, 481, 439, 345, 303	*(epi)gcat-dpd-3,5-dihexoside*
18.45	14	**627**	**465**, 303	303		*Dpd-3,5-diglucoside*
19.42	15	**899**	**737**, 575	**575**	557, 449, 423, 329, 287	*(epi)cat-cyd-3,5-dihexoside*
19.96	16	**737**	**575**	557, 449, 407, 313, 271		*(epi)gcat-pgd-3-hexoside*
20.69	17	**915**	**753**, 591	**591**	573, 465, 423, 329, 287	*(epi)gcat-cyd-3,5-dihexose*
21.13	18	**769**	**607**	589, 481, 439, 345, 303		*(epi)gcat-dpd-3-hexoside*
21.48	19	**753**	**591**	573, 465, 423, 329, 287		*(epi)gcat-cyd-3-hexoside*
22.17	20	**611**	**449**, 287	287		*Cyd-3,5-diglucoside*
22.23	21	**753**	**591**	573, 465, 439, 345, 303		*(epi)cat-dpd-3-hexoside*
22.48	22	**627**	**465**, 303	303		Dpd-3,5-dihexoside
22.58	23	**737**	**575**	557, 449, 407, 313, 271		*(epi)gcat-pgd-3-hexoside*
23.16	24	**753**	**591**	573, 465, 439, 345, 303		*(epi)cat-dpd-3-hexoside*
23.33	25	**597**	**465**, 435, 303	303		Dpd-3,5-pentoside-hexoside
24.01	26	**915**	**753**, 591	**591**	573, 465, 439, 345, 303	*(epi)cat-dpd-3,5-dihexose*
24.19	27	**883**	**721**, 559	**559**	541, 433, 423, 329, 287	*(epi)afz-cyd-3,5-dihexoside*
24.56	28	**737**	**575**	557, 449, 423, 329, 287		*(epi)cat-cyd-3-hexoside*
25.08	29	**595**	**433**, 271	271		*Pgd-3,5-diglucoside*
25.17	30	**753**	**591**	573, 465, 439, 345, 303		*(epi)cat-dpd-3-hexoside*
26.05	31	**899**	**737**, 575	**575**	557, 449, 423, 329, 287	*(epi)cat-cyd-3,5-dihexoside*
26.51	32	**465**	303			*Dpd-3-glucoside*
26.61	33	**581**	**449**, 419, 287	287		*Cyd-3,5-pentoside-hexoside*
26.78	34	**721**	**559**	541, 433, 407, 313, 271		*(epi)cat-pgd-3-hexoside*
26.87	35	**737**	**575**	557, 449, 423, 329, 287		*(epi)cat-cyd-3-hexoside*
27.33	36	**611**	**465**, **449**, 303, 287	465 → 303		Dpd-3-rutinoside
	37			449 → 287		Cyd-3,5-caffeoyl-hexoside
27.72	38	**627**	**465**, 303	303		Dpd-3,5-caffeoyl-hexoside
28.49	39	**753**	**591**	573, 423		Cyd-3-hexoside-(epi)gcat
28.83	40	**721**	**559**	541, 433, 423, 329, 287		*(epi)afz-cyd-3-hexoside*
28.91	41	**737**	**575**	557, 449, 439, 345, 303		*(epi)afz-dpd-3-hexoside*
29.12	42	**449**	287			*Cyd-3-glucoside*
29.82	43	**565**	**433**, 403, 271	271		Pgd-3,5-pentoside-hexoside
29.95	44	**595**	**449**, 287	287		*Cyd-3-rutinoside*
30.65	45	**595**	**433**, 271	271		Pgd-3,5-caffeoyl-hexoside
30.83	46	**449**	287			Cyd-3-hexoside
30.99	47	**705**	**543**	525, 417, 407, 313, 271		*(epi)afz-pgd-3-hexoside*
31.54	48	**721**	**559**	541, 433, 423, 329, 287		*(epi)afz-cyd-3-hexoside*
31.76	49	**433**	271			*Pgd-3-glucoside*
32.83	50	**435**	303			Dpd-3-pentoside
32.98	51	**611**	**449**, 287	287		Cyd-3,5-caffeoyl-hexoside
33.62	52	**705**	**543**	525, 417, 407, 313, 271		*(epi)afz-pgd-3-hexoside*
34.02	53	**611**	**449**, 287	287		Cyd-3,5-caffeoyl-hexoside
35.23	54	**419**	287			*Cyd-3-pentoside*
35.78	55	**419**	287			*Cyd-3-pentoside*
35.81	56	**737**	**575**	557, 423		*Cyd-3-hexoside-(epi)cat*
37.18	57	**403**	271			Pgd-3-pentoside
39.10	58	**465**	303			Dpd-caffeoyl
39.54	59	**721**	**559**	541, 423		*Cyd-3-hexoside-(epi)afz*
44.90	60	**449**	287			Cyd-caffeoyl
46.15	61	**627**	**465**, 303	303		Dpd-3,5-caffeoyl-hexoside
46.83	62	**611**	303			Dpd-3-(p-coumaroyl)hexoside
47.33	63	**611**	**449**, 287	287		Cyd-3,5-caffeoyl-hexoside
47.61	64	**595**	287			Cyd-3-(p-coumaroyl)hexoside
48.37	65	**465**	303			Dpd-caffeoyl

Only those ions with relative abundance greater than 10% are shown. *Abbreviations used*: dpd, delphinidin; cyd, cyanidin; pgd, pelargonidin; (epi)gcat, (epi)gallocatechin; (epi)cat, (epi)catechin; (epi)afz, (epi)afzelechin; Rt, retention time; $[M]^+$, molecular mass under positive ionization conditions; *m*/*z*, mass-to-charge ratio; MS^n, tandem mass spectrometry level reached. Each successive MS^n analysis applies on the ion shown in bold in the preceding column, and the result is given in its own column.

[a] Components in italic have been previously reported in pomegranate juice (Seeram et al., 2006; Sentandreu et al., 2010, 2012; Fischer et al., 2011).

($[M]^+$-162), 449 ($[M]^+$-324) and 287 ($[M]^+$-486), thus indicating a tri-hexoside derivative of cyanidin. As in the above case, this MS^2 fragmentation is compatible with the structure of cyanidin-3-diglucoside-5-glucoside (Wu and Prior, 2005), but the MS^3 of the daughter ion at *m*/*z* 611 yielded ions at *m*/*z* 449 ($[M]^+$-162-162) and at *m*/*z* 287 ($[M]^+$-162-162-162), thus indicating a tri-substituted hexoside derivative of cyanidin. Again, no one free tri-substituted hexoside derivative of cyanidin has been reported

in bibliography from natural sources, although Yoshitama and Abe (1977) detected cyanidin-3,7,3′-trihexoside after deacetylation of an extract from the red petals of Cirenaria, as well as Tatsuzawa et al. (1994) when studying the red flowers of Cattleya and Laelia (*xLaeliocattleya* cv mini purple).

3.1.3. Di-substituted anthocyanidin derivatives

In addition to the major and well-known 3,5-diglucoside derivatives of delphinidin (peak 14), cyanidin (peak 20) and pelargonidin (peak 29), some additional di-substituted "hexoside" derivatives of delphinidin (peaks 5, 9, 22, 38 and 61), cyanidin (peaks 37, 51, 53 and 63) and pelargonidin (peak 45) were also detected. These components exhibited the same characteristic fragmentation pattern than the known di-substituted hexoside derivatives of anthocyanidins, that is, the loss of two consecutive 162 amu and the releasing of the characteristic ion of the aglycone. Therefore, they would correspond to different substitutions of the anthocyanidins with galactose/glucose/caffeoyl moieties, located at different positions within the aglycone structure (normally at positions 3 and 5).

Moreover, several di-substituted glycoside derivatives of anthocyanidins were also detected, namely delphinidin-3,5-pentoside-hexoside (peak 25), the previously reported (Fischer et al., 2011) cyanidin-3,5-pentoside-hexoside (peak 33), cyanidin-3,5-caffeoyl-hexoside (peak 37), and pelargonidin-3,5-pentoside-hexoside (peak 43).

3.1.4. Mono-substituted anthocyanidin derivatives

In addition to the major and well-known 3-glucoside derivatives of delphinidin (peak 32), cyanidin (peak 42) and pelargonidin (peak 49), some other minor "hexoside" derivatives of delphinidin (peaks 58 and 65) and cyanidin (peaks 46 and 60) were also detected. With respect to other mono-glycoside derivatives, the following components were also detected: delphinidin-3-pentoside (peak 50), the previously reported (Fischer et al., 2011) cyanidin-3-pentoside (peaks 54 and 55), and pelargonidin-3-pentoside (peak 57).

Moreover, the following mono-substituted "diglycoside" derivatives of anthocyanidins were detected: delphinidin-3-rutinoside (peak 36), the previously reported (Seeram et al., 2006; Fischer et al., 2011) cyanidin-3-rutinoside (peak 44), delphinidin-3-(*p*-coumaroyl)hexoside (peak 62) and cyanidin-3-(*p*-coumaroyl)-hexoside (peak 64).

The molecular ion corresponding to peaks 62 and 64 was *m*/*z* 611 and 595, respectively. Although these *m*/*z* values are typical for cyanidin and pelargonidin "dihexoside" derivatives, their MS^2 analysis revealed a loss of 308 amu and the releasing of the delphinidin and cyanidin aglycones at *m*/*z* 303 and 287, respectively. These data, in conjunction with the lacking of the typical ion for anthocyanidin rutinosides at *m*/*z* ($[M]^+$-146) and the noticeable delay of their retention times, suggested the presence of an acylated hexoside. Since similar results were reported by Wu et al. (2004) when determining the anthocyanins in black currant, peaks 62 and 64 were identified as delphinidin-3-(*p*-coumaroyl)-hexoside and cyanidin-3-(*p*-coumaroyl)hexoside, respectively.

The mass spectrum of the chromatographic peak eluting at 27.33 min showed a single molecular ion at *m*/*z* 611. However, its MS^2 fragmentation yielded daughter ions at *m*/*z* 465, 449, 303 and 287, being the ion at *m*/*z* 449 by far more intense than that at *m*/*z* 465. The main ion fragmentation sequence, *m*/*z* 611 → 449 → 287, was widely documented from the programmed general data dependent MS^n experiments. Therefore, taking into account its delayed retention time, this component was tentatively identified as cyanidin-3,5-caffeoyl-hexoside (peak 37). On the contrary, the other minor sequence, most probably *m*/*z* 611 → 465 → 303, was not documented at all from the same general MS^n experiments. Finally, this sequence was confirmed from the run (III) of the MS^3 specific analysis for the detection of rutinoside derivatives of anthocyanidins, thus allowing the detection of delphinidin-3-rutinoside (peak 36).

3.2. Non-anthocyanins phenolic profiling

Fig. 2A shows the HPLC chromatogram of pomegranate juice acquired at λ = 320 nm, wavelength at which the anthocyanins exhibit a weak absorbance. In this chromatogram, the six major peaks (numbered as in Table 2) correspond to a punicalin derivative (*m*/*z* 1101, peak 19), punicalagin (peaks 49 and 57), ellagic acid-hexoside dimmer (peak 62), granatin B (peak 79) and ellagic acid (peak 83). As in the case of anthocyanins, a zooming of this chromatogram, as shown in Fig. 2B, reveals the presence of a rather large number of other components that also absorbs at this wavelength, thus indicating that additional non-anthocyanins phenolic components are also present in pomegranate juice, but at lower concentrations. Table 2 lists the 86 non-anthocyanin phenols determined in this work, as well as their chromatographic retention times and MS^n fragmentation patterns.

As pointed out by Fischer et al. (2011), the major non-anthocyanin phenolic component of pomegranate juice is punicalagin (peaks 49 and 57), whose mass spectrum exhibit a molecular ion at *m*/*z* 1083, but also a minor one at *m*/*z* 541, which corresponds to the double charged molecular ion. Some other minor components, namely peaks 29, 33 and 34, also exhibited a deprotonated molecular ion at *m*/*z* 1083, but the ion corresponding to the double charged deprotonated molecular ion at *m*/*z* 541 was lacking in their corresponding mass spectrum. Moreover, the MS^n fragmentation pattern of these components was clearly different

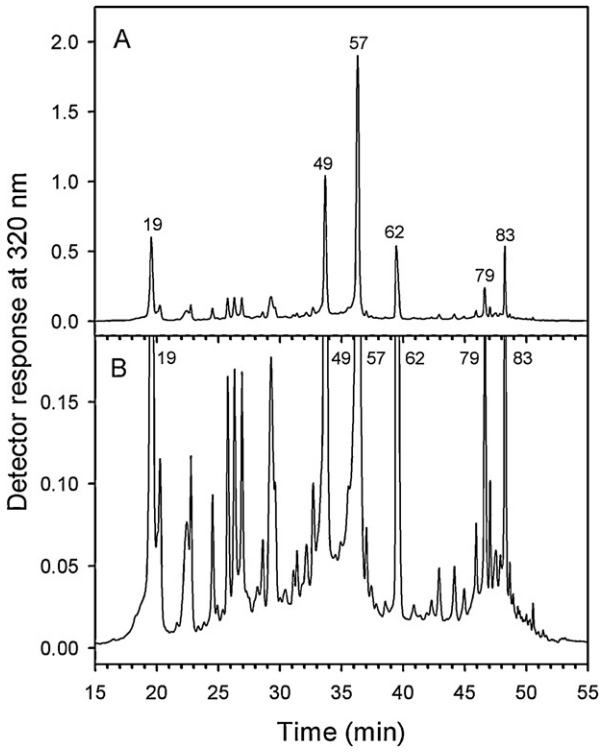

Fig. 2. HPLC chromatogram of pomegranate juice acquired at λ = 320 nm. (A) Normalized standard chromatogram showing the major non-anthocyanin phenolic components: a punicalin derivative (peak 19), punicalagin (peaks 49 and 57), ellagic acid-hexoside dimmer (peak 62), granatin B (peak 79) and ellagic acid (peak 83). (B) Zoomed chromatogram showing additional minor non-anthocyanin phenolic components.

Table 2
Non-anthocyanin phenols detected in pomegranate juice.

Rt (min)	Peak no.	$[M-H]^-$ (*m/z*)	MS^2	MS^3	Identification[a]
5.20	1	**191**	173, **111**	67	Citric acid
5.27	2	**481**	**301**, 275	301, 257, 229	*HHDP-hexoside*
5.86	3	**649**	497, **301**	301, 257, 229	*Lagerstannin C*
6.38	4	**801**	**757**, 481, 301, 275	481, 301, 275	Ellagitannin
6.54	5	**481**	**301**, 275	301, 257, 229	*HHDP-hexoside*
7.03	6	**537** (FA)	491, **329**, 167	209, 167	Vanillic acid-dihexoside
7.52	7	**331**	271, 211, 193, **169**, 151, 125	125	*Monogalloyl-hexoside*
8.47	8	**331**	**271**, 169	169	*Monogalloyl-hexoside*
8.83	9	**801**	**757**, 481, 301, 275	481, 301, 275	Ellagitannin
9.76	10	**969**	**925**, 881, 623, 481, 399	881, 579, 481, 399, 301	Ellagitannin
11.17	11	**331**	**271**	211, 169, 125	*Monogalloyl-hexoside*
13.48	12	**633**	**301**, 275, 249	301, 257, 229	*Galloyl-HHDP-hexoside*[b]
13.78	13	**805**	**643**, 625, 481, 463	517, 481, 463, 355, 301, 283	Ellagitannin
13.86	14	**649**	497, **301**	301, 257, 229	*Lagerstannin C*
16.45	15	**649**	**605**, 301	481, 421, 301	Ellagitannin
17.52	16	**483**	**331**, 313, 169	271, 211, 193, 169	*Digalloyl-hexoside*
18.70	17	**643**	481, **463**, 355, 301, 283	301, 300, 283	Ellagitannin
19.12	18	**625**	**463**, 301	301, 257, 191	*Ellagic acid-dihexoside*
19.95	19	**1101**	1057, **781**, 721, 601	721, 601	Punicalin derivative
20.15	20	**633**	**301**, 275, 249	301, 257, 229	*HHDP-galloylhexoside*[b]
20.34	21	**803**	**759**, 483, 275	483, 331, 275	Gallotannin
20.55	22	**1101**	1057, **781**, 721, 601	721, 601	Punicalin derivative
20.97	23	**329**	269, 209, 181, **167**	152, 123, 108	*Vanillic acid-4-O-hexoside*
21.18	24	**649**	497, **301**	301, 257, 229	*Lagerstannin C*
22.84	25	**643**	**481**	355, 319, 301, 257, 193, 175	Ellagitanin
23.23	26	**483**	**331**, 313, 169	271, 211, 193, 169	*Digalloyl-hexoside*
23.47	27	**341**	**179**, 135	135	*Caffeic acid-hexoside*
24.54	28	**783**	481, **301**, 275	301, 257, 229	*Pedunculagin I*
24.64	29	**1083**	1065, 1021, 959, 807, **601**, 575	301, 299	Gallagyl ester
25.55	30	**783**	481, **301**, 275	301, 257, 229,	*Pedunculagin I*
25.85	31	**341**	**179**, 161, 135	135	*Caffeic acid-hexoside*
25.93	32	**633**	421, **301**, 275	301, 275, 229	*Galloyl-HHDP-hexose*[b]
25.97	33	**1083**	1021, 1003, 959, 807, 721, **601**, 575	583, 301, 299, 271	Gallagyl ester
26.41	34	**1083**	1065, 1021, **807**, 721, 601, 575	763, 601, 575, 549, 425, 301, 299	Gallagyl ester
26.58	35	**463**	**301**, 300	300, 283, 257, 229	*Ellagic acid-hexoside*
27.54	36	**803**	**759**, 483, 275	483, 331, 275	Gallotannin
27.62	37	**933**	915, 781, **721**, 601, 451	601	Galloyl-gallgyl-hexoside (Pedunculagin III)
27.99	38	**1415**	1397, 933, **783**, 763, 633, 613	721, 645, 481, 341, 301	Pedunculagin I derivative
28.67	39	**933**	781, **721**, 601	601	Galloyl-gallagyl-hexoside (Pedunculagin III)
29.67	40	**469**	**425**	425, 407, 300	*Valoneic acid bilactone*
30.05	41	**951**	**907**	783, 481, 301	Pedunculagin I derivative
30.34	42	**341**	281, 251, 221, **179**, 135	135	*Caffeic acid-hexoside*
30.67	43	**461** (FA)	**415**	269, 161	Apigenin-rhamnoside
31.11	44	**325**	187, **163**, 145, 119	119	Coumaric acid-hexoside
31.53	45	**633**	481, 421, **301**, 275	301, 257, 229	*Galloyl-HHDP-hexoside*[b]
31.88	46	**783**	481, **301**, 275	301, 257, 229	*Bis-HHDP-hexoside (pedunculagin I)*
32.44	47	**951**	**907**	889, 783, 605, 481, 301, 271	Ellagitannin
32.66	48	**1265**	1247, 933, **783**, 781, 763, 745, 481	481, 421, 301, 275, 257, 229	Pedunculagin I derivative
33.71	49	**1083**/541	**781**, 721, **601**, 575	781 → 721, 601, 299 601 → 299, 271	*Punicalagin*
34.39	50	**951**	**907**	889, 783, 605, 481, 301, 271	Ellagitannin
34.51	51	**291**	**247**	203	*Brevifolin carboxylic acid*
34.66	52	**1265**	1247, 933, **783**, 763, 745	301, 275, 245, 229	Pedunculagin I derivative
35.38	53	**633**	**301**, 275	301, 275, 229	*Galloyl-HHDP-hexoside*[b]
35.55	54	**1415**	1397, 933, **783**, 763, 633, 613	481, 359, 301, 275, 257	Pedunculagin I derivative
35.83	55	**799**	781, **479**, 301	461, 451, 435, 299, 287, 273	*Granatin A/Lagerstannin A*
35.96	56	**785**	633, 615, 483, **301**	301, 257, 229	*Digalloyl-HHDP-hexoside*
36.21	57	**1083**/541	**781**, 721, **601**, 575	781 → 721, 601, 299 601 → 299, 271	*Punicalagin*
36.82	58	**1417**	**785**, 765, 633, 613, 451	483, 419, 401, 301, 275, 231	Ellagitannin
37.32	59	**1083**	781, 721, **601**, 575	299, 271	*Punicalagin*
37.67	60	**801**	649, 499, 347, **301**	301, 257	*Punigluconin*
38.86	61	**801**	649, 499, 347, **301**	301 → 301, 257 649 → 497, 301	*Punigluconin*
39.12	62	**463/927**	463 → **301** 927 → 463, 301	301 → 301, 257, 229	Ellagic acid-hexoside dimmer
39.16	63	**633**	463, **301**, 275	301, 257, 229	*Galloyl-HHDP-hexoside*[b]
39.24	64	**463**	**301**	301, 257, 229	*Ellagic acid hexoside*
39.28	65	**935**	659, **633**, 571, 301	571, 301	*Galloyl-bis-HHDP-hexoside (casuarinin)*
40.61	66	**1085**	**933**, 915, 719, 601, 575, 549	781,721, 601, 549, 299	Digalloyl-gallagyl-hexoside
41.30	67	**783**	**765**, 301, 275	597, 301, 275	*Pedunculagin I*

Table 2 (*Continued*)

Rt (min)	Peak no.	$[M-H]^-$ (m/z)	MS^2	MS^3	Identification[a]
41.39	68	**507**	**345**, **327**, 315	327 → 312, 296, 283, 268 345 → 327, 315	Syringetin-hexoside
41.98	69	**785**	767, 633, 483, 419, **301**	301, 257, 229	*Digalloyl-HHDP-hexoside*
42.77	70	**1085**	783, **765**	597, 301, 275	Tri-HHDP-hexoside
43.01	71	**783**	**765**, 481, **301**	765 → 746, 301, 299 301 → 275, 229	*Pedunculagin I*
43.97	72	**1085**	783, **765**	597, 301	Tri-HHDP-hexoside
44.79	73	**468**	**425**	301, 299	*Valoneic acid bilactone*
45.34	74	**953**	**935**, 463, 301	891, 463, 343, 301	Ellagitannin
45.54	75	**785**	633, 615, 483, 419, **301**, 275, 249	301, 257, 229	*Digalloyl-HHDP-hexoside*
45.65	76	**615**	**463**, 301	301, 257	Ellagic acid-galloyl-hexoside
45.68	77	**433**	**301**	301, 300, 257, 229	*Ellagic acid-pentoside*
45.80	78	**507**	489, **345**, **327**, 315	327 → 312, 296, 283, 268 345 → 327, 315	Syringetin-hexoside
46.01	79	**951**	**933**, 631, **613**, 301	933 → 631, 613, 301 613 → 301, 299	*Granatin B*
46.39	80	**447**	**301**, 300	301, 300, 257, 229	*Ellagic acid-rhamnoside*
46.50	81	**433**	300, **301**	300, 301	*Ellagic acid-pentoside*
47.45	82	**601**	**299**, 271	271	Gallagic acid
48.08	83	**301**	**301**, 257, 229	301, 257, 229	*Ellagic acid*
49.98	84	**609**	**463**, 445, **301**	301 → 257 463 → 301	Ellagic acid-(*p*-coumaroyl)hexoside
52.32	85	**449**	**431**, 287	287	*Dihydrokaempferol-hexoside*
52.82	86	**593**	445, **301**	301, 300, 257	Ellagitannin

Only those ions with a relative abundance greater than 10% are shown. (FA), detected as formic acid adduct. Each successive MS^n analysis applies on the ions shown in bold in the preceding column, and the result is given in its own column. *Abbreviations used*: Rt, retention time; $[M-H]^-$, molecular mass under negative ionization conditions; m/z, mass-to-charge ratio; MS^n, tandem mass spectrometry level reached; HHDP, hexahydroxydiphenic acid.

[a] Components in italic have been previously reported by Fischer et al. (2011).

[b] The ion at m/z 633 could correspond to corilagin, strictinin or punicacortein A/B.

from that of punicalagin (see Table 2), and the presence as main fragments of the specific daughter ions at m/z 601 and m/z 301/299 clearly suggested for them a structure of gallagyl derivative, most probably ester.

When the ion at m/z 1101 was searched across the total ion current (TIC) chromatogram (Fig. 3A), two significant peaks were found (Fig. 3B), one of them corresponding to the aforementioned

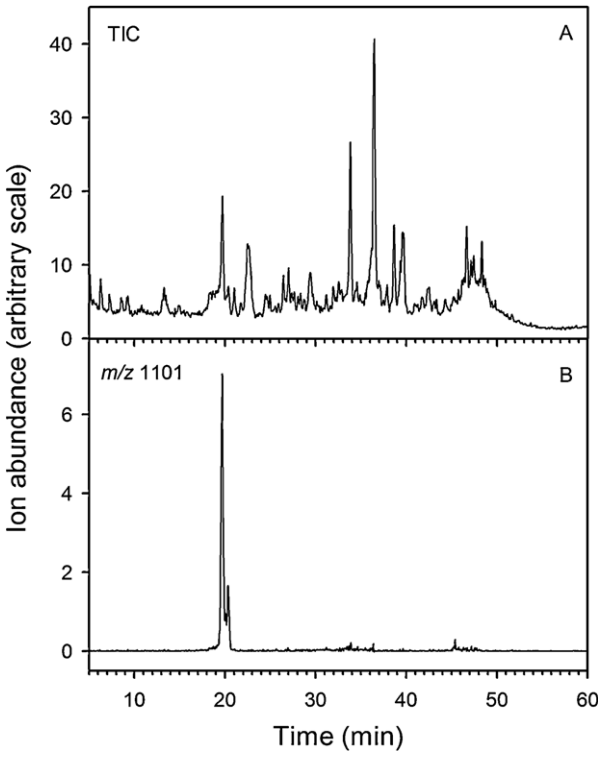

Fig. 3. (A) Total ion current (TIC) chromatogram of pomegranate juice under negative ESI ionization mode. (B) Single ion monitoring (SIM) chromatogram corresponding to the extraction of the ion with m/z 1101 from the TIC chromatogram.

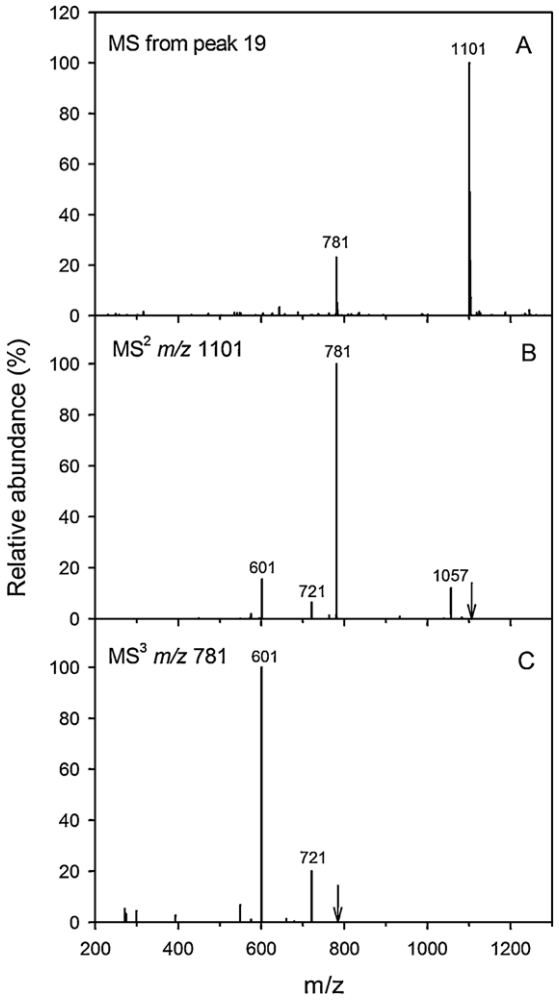

Fig. 4. MS^n analysis of the parent ion at m/z 1101 (peaks 19 and 22, punicalin derivatives). (A) Full MS of the ion at m/z 1101; (B) MS^2 of the ion at m/z 1101; (C) MS^3 of the major daughter ion at m/z 781 (arrows show targeted m/z's for the MS^2 and MS^3 analyses).

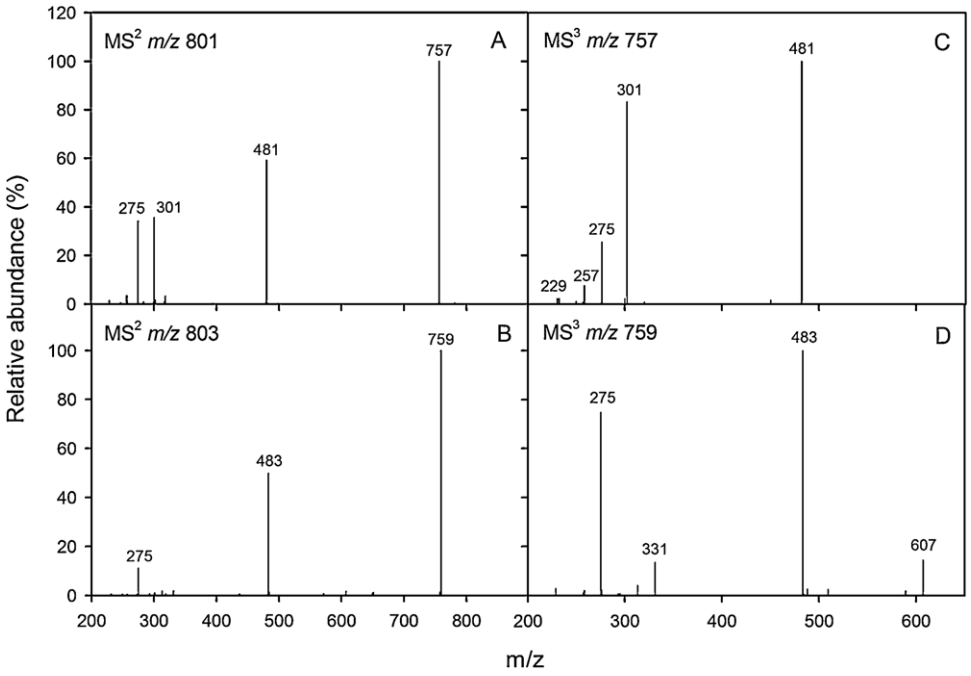

Fig. 5. MS^n analysis of the parent ions at *m/z* 801 (peaks 4 and 9) and 803 (peaks 21 and 36). (A) MS^2 of the parent ion at *m/z* 801; (B) MS^2 of the parent ion at *m/z* 803; (C) MS^3 of the major daughter ion at *m/z* 757 from the MS^2 of the parent ion at *m/z* 801; (D) MS^3 of the major daughter ion at *m/z* 759 from the MS^2 of the parent ion at *m/z* 803.

peak 19 and the other to the minor peak 22. The MS spectrum of both compounds were very similar, Fig. 4A, showing a deprotonated molecular ion at *m/z* 1101 and a minor one at *m/z* 781, thus suggesting their close relationship with punicalin. Hence, the MS^2 of *m/z* 1101 (Fig. 4B) yielded ions at *m/z* 1057, 781, 721 and 601, whilst the MS^3 of the main fragment at *m/z* 781 (Fig. 4C) yielded ions at *m/z* 721 and 601. Borges et al. (2010) cited the presence of a component with a rather similar MS^n fragmentation pattern, excepting that the ion at *m/z* 1057 ($[M-H]^-$-44) (loss of a carboxyl group) was lacking, and suggested for it a structure of punicalagin derivative. In our case, however, taking into account the presence of the ions at *m/z* 1057, 721 and 601, as well as that the main fragment ion from the MS^2 of the deprotonated molecular ion was *m/z* 781 ($[M-H]^-$-44-276) for both components, it seems more appropriate to tentatively identify them as punicalin derivatives. On the other hand, the observed difference of 276 amu between the ions at *m/z* 1057 and *m/z* 781 (the punicalin ion) deserves special attention. This mass most probably correspond to 3,4,8,9,10-pentahydroxy-dibenzo[b,d]pyran-6-one which, similarly to breviofolin and chebullic acid, is a biosynthetic transformation product from hexahydroxydiphenic acid (Nawwar and Souleman, 1984). Moreover, when Ito (2011) identified the metabolites formed during the metabolism of geraniin in rats, proposed 3,4,8,9,10-pentahydroxy-dibenzo[b,d]pyran-6-one to be a direct metabolism product of ellagic acid which, in its turn, comes from gallic acid in the shikimic acid biosynthetic pathway (Ishikura et al., 1984). Finally, Nawwar et al. (1994) cited the presence of this compound in the leaves of Egyptian pomegranates. In spite of this, 3,4,8,9,10-pentahydroxy-dibenzo[b,d]pyran-6-one could not be detected as a free component in pomegranate juice.

Although the gallagyl group is a part of the chemical structure of many of the phenols that are commonly found in pomegranate juice, such as punicalin and punicalagin derivatives, its presence as free gallagic acid has only been referenced in peel extracts (Zahin et al., 2010). Hence, its detection in pomegranate juice (peak 82) could be due to the pressure applied to the whole fruit during the extraction step. In addition, some gallagic acid derivatives, such as pedunculagin III (peaks 37 and 39) and digalloyl-gallagyl-hexoside (peak 66), were detected for the first time in pomegranate juice.

Similarly to peaks 19 and 22, the main transition from the MS^2 of peaks 4 and 9 (*m/z* 801) and 21 and 36 (*m/z* 803) was the release of a neutral fragment of 44 amu (carboxylic group), yielding major fragments at *m/z* 757 (Fig. 5A) and at *m/z* 759 (Fig. 5B), respectively. Moreover, the main transition from the MS^3 of ions at *m/z* 757 and *m/z* 759 was again the releases of a neutral fragment of 276 amu, yielding major fragments at *m/z* 481 (Fig. 5C) and at *m/z* 483 (Fig. 5D). Although both groups of components shared the fragment at *m/z* 275, the MS^3 of peaks 4 and 9 (Fig. 5C) showed also the presence of ions at *m/z* 301, 257 and 229, clearly suggesting that they were ellagic acid derivatives. In contrast, in the MS^3 of peaks 21 and 36 (Fig. 5D) the ion at *m/z* 301 was lacking, but there were ions at *m/z* 607 (loss of a galloyl group) and at *m/z* 331 (galloyl-hexoside), thus indicating the gallotannin nature of both components. It is remarkable the different behavior of the neutral fragment of 276 amu from peaks 19/22 and peaks 4/9/21/36. Although in all cases a neutral fragment of 44 amu followed by an additional fragment of 276 u were released, the ion at *m/z* 275 could be observed in the MS^n spectra from peaks 4/9/21/36, but not in those from peaks 19/22. A plausible explanation could be that whilst 3,4,8,9,10-pentahydroxy-dibenzo[b,d]pyran-6-one (probably carrying a carboxylic acid) is part of the basic structure of components 4, 9, 21 and 36, and hence it is finally generated during their MS^n fragmentation, it is a mere substituent in components 19 and 22.

Several ellagic acid derivatives were detected as new components in pomegranate juice, such as pedunculagin I derivatives (peaks 38, 41, 48, 52 and 54), a dimmer of ellagic acid-hexoside (peak 62), tri-HHDP-hexoside (peaks 70 and 72), ellagic acid-galloyl-hexoside (peak 76), and ellagic acid-(*p*-coumaroyl)glucoside (peak 84). Moreover, additional components with ellagitannin structure were peaks 10, 13, 17, 25, 47, 50, 58, 74 and 86, all them sharing the ion at *m/z* 301 in their respective MS^n fragmentation patterns. The structures of these later components were determined from the generation of typical ion fragments in their MS^n spectrum, such as *m/z* 783 (pedunculagin I), 633 (HHDP-galloylhexoside), 481

(HHDP-hexoside) and 463 (ellagic acid-hexoside), which came from the loss of different functional groups (mainly hexosides).

It is of major importance to take into account, as stated by Fischer et al. (2011) that the ion at m/z 301 can correspond to both ellagic acid and quercetin. Therefore, it must be checked before the final assignment of a chemical structure to a component. The MS^n of m/z 301 yields fragments at m/z 257, 229 and 185 when it corresponds to ellagic acid and at m/z 179 and 151 when it corresponds to quercetin. Another consideration that must be taken into account is the capacity of HHDP to lactonize to ellagic acid. Hence, the HHDP derivatives, normally glycosides, exhibit a molecular mass 18 u higher than their respective ellagic acid counterparts, such as peaks 5 (m/z 481, HHDP-hexoside) and 37 (m/z 463, ellagic acid-hexoside).

Additionally, some other families of chemicals were also detected, such as phenolic acid derivatives, organic acids and flavonoids. Among them, vanillic acid-dihexoside (peak 6), apigenin-rhamnoside (peak 43), coumaric acid-hexoside (peak 44) and syringetin-hexoside (peaks 68 and 78) were detected for the first time in pomegranate juice.

4. Conclusions

Pomegranate juice has been characterized as a function of its profiles in anthocyanin and non-anthocyanin phenols. An exhaustive methodology of analysis, using different scan modes for mass analysis featured by a conventional three-dimensional ion trap analyzer, has been developed, thus making possible the detection of a total of 151 phenols, 64 not previously reported in pomegranate juice including several cyanidin and pelargonidin tri-hexoside derivatives, which have been detected as free forms from a natural source for the first time.

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