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ARTICLE

LC-DAD-ESI/MS^n Determination of Direct Condensation Flavanol−Anthocyanin Adducts in Pressure Extracted Pomegranate (*Punica granatum* L.) Juice

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Pomegranate (*Punica granatum* L.) juice, obtained by pressure extraction of the whole fruit, has been analyzed for its flavanol−anthocyanin adduct content using reversed-phase liquid chromatography with diode array detection, coupled to mass spectrometry (ion trap) with electrospray ionization (HPLC-DAD−ESI/MS^n), operating in positive ion mode. A total of 35 dimers have been detected, consisting of mono- and disubstituted hexoside derivatives of the adducts between the flavan-3-ols (epi)gallocatechin, (epi)catechin and (epi)afzelechin and the anthocyanidins delphinidin, cyanidin and pelargonidin. In addition, evidence is given for the presence of additional anthocyanin−flavanol adducts in this juice.

KEYWORDS: Pomegranate juice; flavanol−anthocyanin adducts; liquid chromatography; mass spectrometry

INTRODUCTION

Pomegranate (*Punica granatum* L.) is one of the oldest edible fruits that was extensively used in folk medicine of many ancient cultures, as well as believed as a fruit invested of some mystical properties due, in part, to its characteristic deep red color, similar to human blood. The phytochemical composition of pomegranate fruit is rather complex, including ellagitannins, gallotannins, ellagic acids, gallagic acids, catechins, anthocyanins, ferulic acids, quercetins and isoflavones (*1*, *2*). Concerning its main components, the anthocyanins, which as in many other fruits and plants are responsible for its deep red color (*3*), only the six major ones, namely, cyanidin-3-glucoside, delphinidin-3-glucoside, cyanidin-3,5-diglucoside, delphinidin-3,5-diglucoside, pelargonidin-3-glucoside and pelargonidin-3,5-diglucoside, have already been identified and quantified by paper (*4*) and liquid (*5*) chromatography (LC) with diode array detection (DAD). Many studies have demonstrated important pharmacodynamic activities linked to the intake of pomegranate arils or juice (*6*), which are mainly attributed to the antioxidant and antiradical activity of its components (*7*). As a consequence, consumers believe pomegranate and pomegranate juice to be a preferred food to be included in everyday nutrition, thus catapulting pomegranate fruit and juice into a prominent position in international commerce (*8*).

Anthocyanins are able to form adducts with flavanols. Basically, three types of anthocyanin−flavanol adducts have been described, the proposed reaction pathways being the following: (1) direct condensation between the anthocyanin and the flavanol, yielding flavanol−anthocyanin (the flavanol occupies the upper position of the dimer) and anthocyanin−flavanol (the anthocyanin occupies the upper position of the dimer) adducts (*9*−*11*); (2) condensation of the anthocyanin and the flavanol mediated by aldehydes (*12*, *13*), mainly by acetaldehyde thus yielding ethyl-linked adducts (*14*, *15*); (3) cycloaddition reaction at anthocyanin C4-position involving vinylphenol derivatives (*16*), pyruvic acid (*17*) and vinylflavanols (*18*). Practically all these types of adducts were formerly detected in aged red wines (*19*−*22*), and so they were mostly associated with anthocyanin transformations taking place during the maturation and aging of red wines. Recent studies show, however, that certain anthocyanin-derived pigments, mainly direct condensation adducts of the flavanol−anthocyanin type, occur not only in red wines but also in other plants although in very small amounts. Hence, direct condensation flavanol−anthocyanin adducts have been reported in strawberry (*23*), beans (*24*), purple corn (*25*) and black currant (*26*).

Flavanol−anthocyanin adducts in plants are rather difficult to detect and require specialized instrumentation. This is so because these products are usually found at very small concentrations and, in addition, they can coelute with some other major components, mainly anthocyanins, which absorb at the same wavelengths. Hence, it is very unlikely that the HPLC-DAD technique alone could give useful information, and a much more powerful technique such as HPLC-DAD-ESI/MS^n is needed. Even so, the molecular ions of the flavanol−anthocyanin adducts, which normally are very minor ones within the total ion chromatogram (TIC) from the MS of the injected sample, almost do not give noticeable fragments under the soft ESI ionization (*27*). As a consequence, to acquire the mass spectrum of the flavanol−anthocyanin aglycon, which carries the necessary information for its identification (*10*, *11*, *21*, *22*, *24*, *25*, *28*), collision induced dissociation tandem MS/MS spectrometry and MS^n analysis are required.

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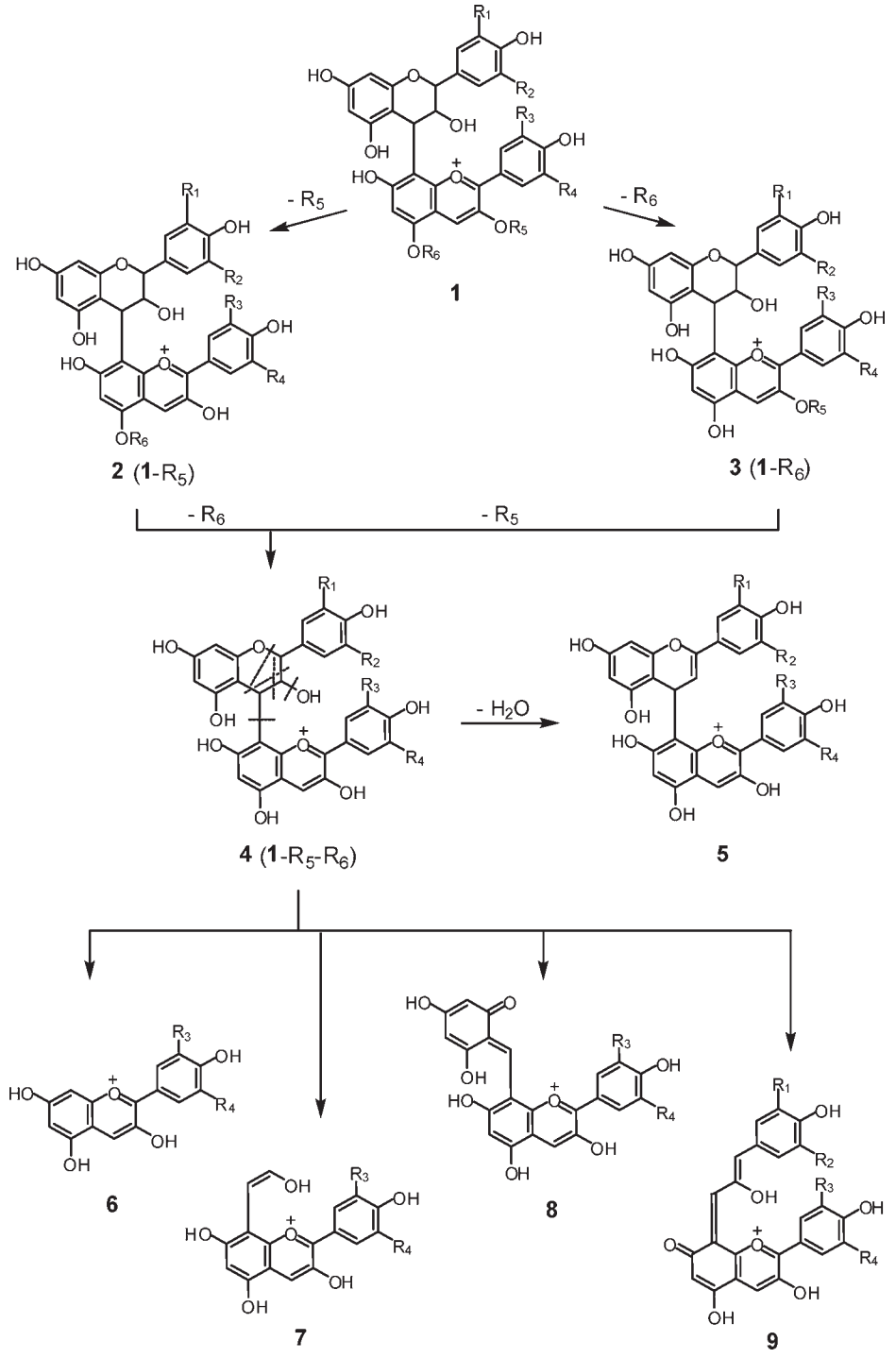

Figure 1. General fragmentation pathway of the flavanol−anthocyanin adducts.

MATERIALS AND METHODS

Reagents and Standards. Spectrophotometric water, acetonitrile and tetrahydrofuran (THF), as well as analytical grade formic acid, were from Scharlab (Scharlab S. L., Barcelona, Spain). Nylon filters (0.45 μm) were from Teknokroma (Teknokroma Ltd., Barcelona, Spain).

Pomegranate Juice. Pomegranate fruits, cultivar 'Wonderful', were grown and imported from Peru. Eight units of fruit averaged a weight of 3.8 kg. Juice was obtained by pressure extraction of the whole fruit, previously cut into halves, using a pressure extractor Europ (Vapfluid, Sant Boi de Llob, Barcelona, Spain) working at an air pressure of 6 kg/cm^2. Yield of juice was 40% (w/w), having the following characteristics: °Brix, 16; pH, 3.1; acidity index, 1.15; and maturity index, 13.9. Aliquots of pomegranate juice were immediately stored at −30 °C until analysis, which was carried out as soon as possible.

Sample Preparation. Before analysis, an aliquot of the pomegranate juice was defrosted at room temperature and darkness in a water bath and centrifuged at 5000 rpm during 5 min; the supernatant was filtered through a 0.45 μm nylon filter and then injected for HPLC-DAD-ESI/MS^n analysis.

HPLC-DAD-ESI/MS^n Analysis. Chromatographic separation was achieved using a Thermo Surveyor Plus HPLC (Thermo Scientific, San Jose, CA), equipped with a quaternary pump, vacuum degasser, temperature-controlled autosampler, and DAD detector. 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 as follows: injection volume, 10 μL; 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); solvent B, acetonitrile/THF/formic acid (97.5:2.0:0.5, v/v);

elution began with 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 5 min and re-equilibration of the column during 20 min. UV/vis absorbance spectra were recorded from 230 to 600 nm, and detection wavelengths were set at 520, 320, and 280 nm.

MS analysis and fragmentation experiments were performed on a ThermoFinningan LCQ Advantage (Thermo Scientific, San Jose, CA) ion trap mass spectrometer equipped with an ESI source which were controlled, as well as the HPLC-DAD and data analysis, using the software Xcalibur loaded into a PC computer. The mass spectrometer was operated in the positive ion mode in the range m/z 150−2000, under the following conditions: source voltage, 3.5 kV; capillary voltage, 9 V; capillary temperature, 300 °C; sheath gas flow, 50; and sweep gas flow, 20. The scan sequence parameters were as follows: AGC off ion time, 5 ms; zoom micro scans, 5; zoom max ion time, 50 ms; full micro scans, 3; full max ion time, 300 ms; SIM micro scans, 5; SIM max ion time, 200 ms; MS^n micro scans, 3; and MS^n max ion time, 600 ms. For monosubstituted flavanol−anthocyanin adducts (+R, most probably at the 3 position on the anthocyanidin), the collision energy for fragmentation was set at 35% for both the MS^2 of the selected parent ion (m/z M^+) and the MS^3 of the selected first daughter ion (m/z $M^+ - R$, the flavanol−anthocyanin aglycon). For disubstituted flavanol−anthocyanin adducts ($+R_1 + R_2$, most probably at the 3 and 5 positions on the anthocyanidin), the collision energy for fragmentation was also set at 35% for the MS^2 of the selected parent ion, the MS^3 of the selected first isotopic daughter ions (m/z $M^+ - R_1 = m/z$ $M^+ - R_2$, since it was $R_1 = R_2$ in all cases) and the MS^4 of the selected flavanol−anthocyanin aglycon (m/z $M^+ - R_1 - R_2$).

RESULTS AND DISCUSSION

As indicated previously, the flavanol−anthocyanin adducts in plants are normally found in small concentrations. As a consequence, their detection and identification require a systematic checking of the ions corresponding to their molecular weights, which are minor ones within the multitude of ions present in the TIC from the MS of the sample. Although in pomegranate juice only the anthocyanidins delphinidin, cyanidin and pelargonidin have been detected, in a preliminary step the molecular ions

Table 1. Characteristic Ions of the Aglycons from the Flavanol−Anthocyanin Adducts Detected in Pomegranate Juice[a]

anthocyanidin	flavan-3-ol	aglycon	M^+ (m/z)	characteristic ions
dpd	(epi)gcat	(epi)gcat-dpd	607	303, 345, 439, 481, 589
	(epi)cat	(epi)cat-dpd	591	303, 345, 439, 465, 573
	(epi)afz	(epi)afz-dpd	575	303, 345, 439, 449, 557
cyd	(epi)gcat	(epi)gcat-cyd	591	287, 329, 423, 465, 573
	(epi)cat	(epi)cat-cyd	575	287, 329, 423, 449, 557
	(epi)afz	(epi)afz-cyd	559	287, 329, 423, 433, 541
pgd	(epi)gcat	(epi)gcat-pgd	575	271, 313, 407, 449, 557
	(epi)cat	(epi)cat-pgd	559	271, 313, 407, 433, 541
	(epi)afz	(epi)afz-pgd	543	271, 313, 407, 417, 525

[a] Abbreviations: dpd, delphinidin; cyd, cyanidin; pgd, pelargonidin; (epi)gcat, (epi)gallocatechin; (epi)cat, (epi)catechin; (epi)afz, (epi)afzelechin.

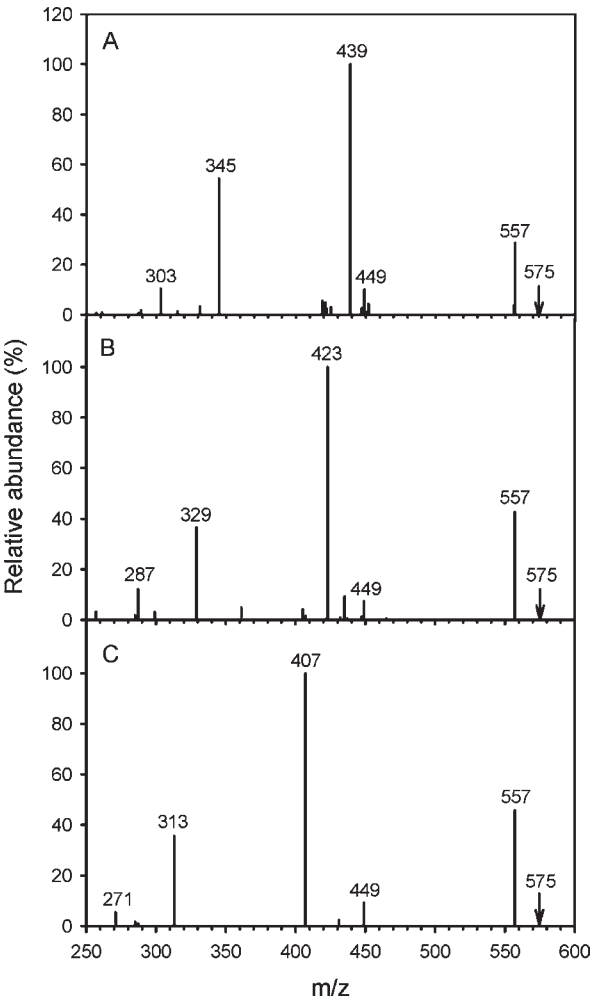

Figure 3. Typical mass spectrum of the aglycons of (epi)afzelechin−delphinidin (**A**), (epi)catechin−cyanidin (**B**), and (epi)gallocatechin−pelargonidin (**C**), obtained from the MS^4 of the selected aglycon ions at m/z 575.

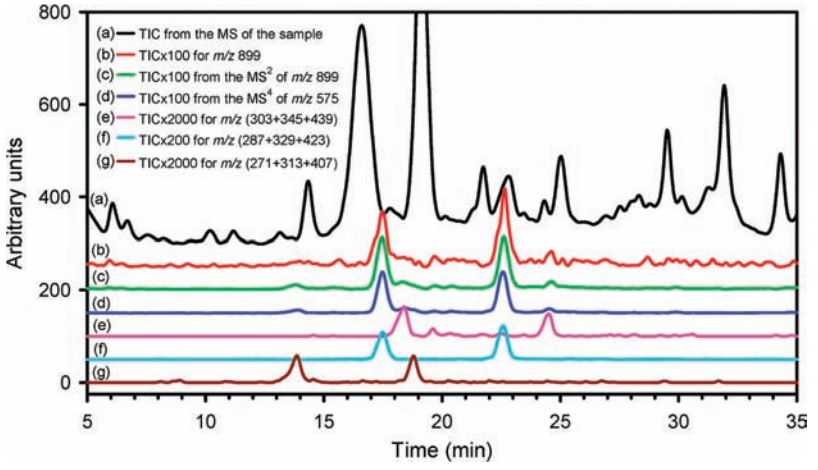

Figure 2. Graphical representation of the TICs obtained during the successive steps for the detection and identification of the flavanol−anthocyanin adducts sharing the molecular weight m/z 899.

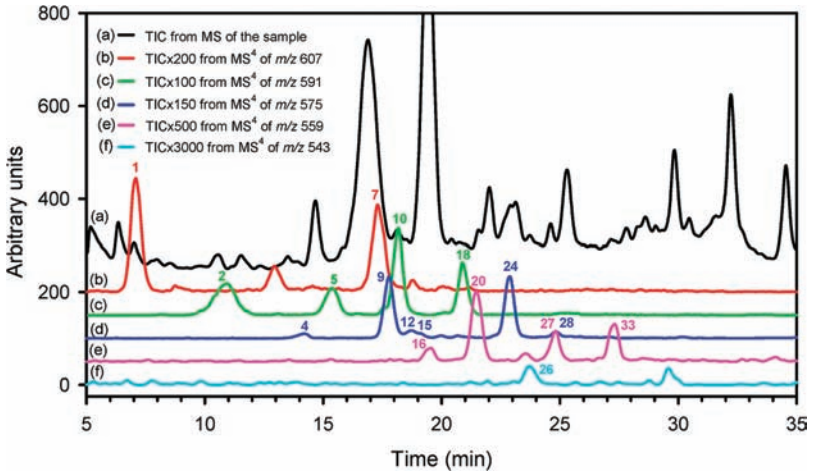

Figure 4. Graphical representation of the TIC from the MS of pomegranate juice, and the TICs from the MS^4 of the selected aglycon ions at *m/z* 607, 591, 575, 559 and 543, corresponding to the flavanol−anthocyanin-3,5-dihexoside adducts with molecular weights at *m/z* 931, 915, 899, 883 and 867, respectively. For additional details about numbered peaks, see **Table 2**.

Table 2. Retention Time, Peak Number, Molecular Weight, Fragmentation Pathway,[a] Characteristic Ions and Identification of the Flavanol−Anthocyanin Adducts Detected in Pomegranate Juice[b]

t_R (min)	peak no.	M^+ (*m/z*)	MS^2	MS^3	MS^4	identification
7.10	1	**931**	**769**, 607	**607**	589, 481, 439, 345, 303	(epi)gcat-dpd-3,5-dihexoside
10.97	2	**915**	**753**, 591	**591**	573, 465, 423, 329, 287	(epi)gcat-cyd-3,5-dihexoside
13.57	3	**769**	**607**	589, 481, 439, 345, 303		(epi)gcat-dpd-3-hexoside
14.18	4	**899**	**737**, 575	**575**	557, 449, 407, 313, 271	(epi)gcat-pgd-3,5-dihexoside
15.39	5	**915**	**753**, 591	**591**	573, 465, 439, 345, 303	(epi)cat-dpd-3,5-dihexoside
15.86	6	**753**	**591**	573, 465, 423, 329, 287		(epi)gcat-cyd-3-hexoside
17.27	7	**931**	**769**, 607	**607**	589, 481, 439, 345, 303	(epi)gcat-dpd-3,5-dihexoside
17.63	8	**737**	**575**	557, 449, 407, 313, 271		(epi)gcat-pgd-3-hexoside
17.77	9	**899**	**737**, 575	**575**	557, 449, 423, 329, 287	(epi)cat-cyd-3,5-dihexoside
18.20	10	**915**	**753**, 591	**591**	573, 465, 423, 329, 287	(epi)gcat-cyd-3,5-dihexoside
18.50	11	**769**	**607**	589, 481, 439, 345, 303		(epi)gcat-dpd-3-hexoside
18.70	12	**899**	**737**, 575	**575**	557, 449, 439, 345, 303	(epi)afz-dpd-3,5-dihexoside
18.84	13	**753**	**591**	573, 465, 423, 329, 287		(epi)gcat-cyd-3-hexoside
19.10	14	**753**	**591**	573, 465, 439, 345, 303		(epi)cat-dpd-3-hexoside
19.13	15	**899**	**737**, 575	**575**	557, 449, 407, 313, 271	(epi)gcat-pgd-3,5-dihexoside
19.49	16	**883**	**721**, 559	**559**	541, 433, 407, 313, 271	(epi)cat-pgd-3,5-dihexoside
19.85	17	**737**	**575**	557, 449, 407, 313, 271		(epi)gcat-pgd-3-hexoside
20.86	18	**915**	**753**, 591	**591**	573, 465, 439, 345, 303	(epi)cat-dpd-3,5-dihexoside
20.91	19	**737**	**575**	557, 449, 423, 329, 287		(epi)cat-cyd-3-hexoside
21.43	20	**883**	**721**, 559	**559**	541, 433, 423, 329, 287	(epi)afz-cyd-3,5-dihexoside
21.81	21	**753**	**591**	573, 465, 439, 345, 303		(epi)cat-dpd-3-hexoside
22.52	22	**737**	**575**	557, 449, 439, 345, 303		(epi)afz-dpd-3-hexoside
22.70	23	**721**	**559**	541, 433, 407, 313, 271		(epi)cat-pgd-3-hexoside
22.85	24	**899**	**737**, 575	**575**	557, 449, 423, 329, 287	(epi)cat-cyd-3,5-dihexoside
23.31	25	**737**	**575**	557, 449, 423, 329, 287		(epi)cat-cyd-3-hexoside
23.69	26	**867**	**705**, 543	**543**	525, 417, 407, 313, 271	(epi)afz-pgd-3,5-dihexoside
24.82	27	**883**	**721**, 559	**559**	541, 433, 407, 313, 271	(epi)cat-pgd-3,5-dihexoside
24.86	28	**899**	**737**, 575	**575**	557, 449, 439, 345, 303	(epi)afz-dpd-3,5-dihexoside
24.88	29	**721**	**559**	541, 433, 423, 329, 287		(epi)afz-cyd-3-hexoside
25.07	30	**721**	**559**	541, 433, 407, 313, 271		(epi)cat-pgd-3-hexoside
25.23	31	**737**	**575**	557, 449, 439, 345, 303		(epi)afz-dpd-3-hexoside
27.07	32	**705**	**543**	525, 417, 407, 313, 271		(epi)afz-pgd-3-hexoside
27.35	33	**883**	**721**, 559	**559**	541, 433, 423, 329, 287	(epi)afz-cyd-3,5-dihexoside
27.77	34	**721**	**559**	541, 433, 423, 329, 287		(epi)afz-cyd-3-hexoside
29.99	35	**705**	**543**	525, 417, 407, 313, 271		(epi)afz-pgd-3-hexoside

[a] 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. [b] Abbreviations: dpd, delphinidin; cyd, cyanidin; pgd, pelargonidin; (epi)gcat, (epi)gallocatechin; (epi)cat, (epi)catechin; (epi)afz, (epi)afzelechin.

corresponding to the mono- (pentoside, rhamnoside, hexoside, sophoroside, sambubioside, etc.) and disubstituted (pentoside, rhamnoside, hexoside, sophoroside, sambubioside, etc.) adducts between the flavan-3-ols (epi)gallocatechin, (epi)catechin and (epi)afzelechin and all the above anthocyanidins plus peonidin, petunidin and malvidin were extracted from the TIC of the MS of the sample. Only when the anthocyanidin was delphinidin, cyanidin or pelargonidin and the substituents were hexoses, noticeable peaks were found. Hence, only the following parent molecular weights were considered: *m/z* 705 and 867 corresponding,

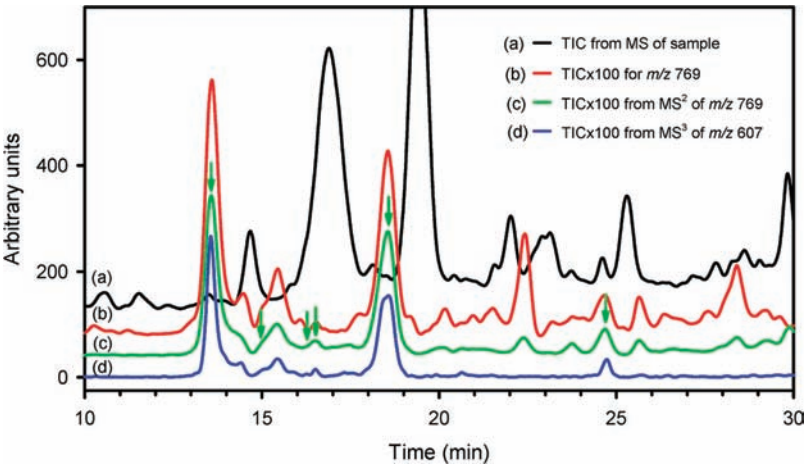

Figure 5. Graphical representation of the TIC from the MS of pomegranate juice, and the TICs obtained during the successive step for the study of the parent ion at *m/z* 769. Peaks in the TIC from the MS^2 of the selected parent ion, plot (**c**), whose mass spectrum fulfills the expected fragmentation are marked with an arrow.

respectively, to the putative mono- and disubstituted hexoside derivatives of the (epi)afzelechin−pelargonidin adducts; *m/z* 721 and 883 corresponding, respectively, to the putative mono- and disubstituted hexoside derivatives of the (epi)afzelechin−cyanidin and (epi)catechin−pelargonidin adducts; *m/z* 737 and 899 corresponding, respectively, to the putative mono- and disubstituted hexoside derivatives of the (epi)afzelechin−delphinidin, (epi)catechin−cyanidin and (epi)gallocatechin−pelargonidin adducts; *m/z* 753 and 915 corresponding, respectively, to the putative mono- and disubstituted hexoside derivatives of the (epi)catechin−delphinidin and (epi)gallocatechin−cyanidin adducts; and *m/z* 769 and 931 corresponding, respectively, to the putative mono- and disubstituted hexoside derivatives of the (epi)gallocatechin−delphinidin adducts.

Positive detection of a substituted flavanol−anthocyanin adduct requires the acquisition of the mass spectrum of its aglycon. Several authors have deciphered the fragmentation pathway of the aglycons and demonstrated that the resulting mass spectra exhibit a pattern of characteristic ions as a function of the implied flavanol and anthocyanidin (*10*, *11*, *21*, *22*, *24*, *25*, *28*). In short, this fragmentation pathway and series of characteristic ions can be summarized and systematized as indicated in **Figure 1**.

As can be seen, after the loss of R_5, in the case of monosubstituted adducts (R_6 = H), or R_5 and R_6, in the case of disubstituted adducts, the resulting flavanol−anthocyanin aglycon (**4**) yields a mass spectrum containing five characteristic ions (**5**−**9**), three of them (**6**−**8**) depending exclusively on the anthocyanidin and the other two (**5** and **9**) on both the flavanol and the anthocyanidin. For instance, assuming that the flavan-3-ol is (epi)catechin (R_1 = OH and R_2 = H) and the anthocyanidin is cyanidin (R_3 = OH and R_4 = H), then the mass spectrum of the (epi)catechin−cyanidin aglycon (**4**, *m/z* 575) must contain the three specific ions for cyanidin, namely, *m/z* 287 (**6**, cyanidin), *m/z* 329 (**7**, cyanidin + 42) and *m/z* 423 (**8**, cyanidin + 136), and the two ions from the whole aglycon, *m/z* 449 [**9**, (epi)catechin−cyanidin − 126] and *m/z* 557 [**5**, (epi)catechin−cyanidin − 18]. **Table 1** gives the characteristic ions from the aglycons of the different flavanol−anthocyanin adducts present in pomegranate juice.

Flavanol−Anthocyanin-3,5-dihexosides. As an example, let us considerer in detail the different steps carried out for the study of the parent ion at *m/z* 899 (**1**) which, as indicated previously, could correspond to the 3,5-dihexoside derivatives of the adducts (epi)gallocatechin−pelargonidin, (epi)catechin−cyanidin and (epi)afzelechin−delphinidin, but also to the 3-sophoroside, 3-laminaribioside or 3-gentiobioside derivatives of these same adducts, as well as to the 3-hexoside-5-pentoside derivatives of the adducts (epi)gallocatechin−cyanidin and (epi)catechin−delphinidin.

In **Figure 2**, plot (**a**) corresponds to the TIC from the MS of pomegranate juice; plot (**b**) corresponds to the 100-fold magnified TIC for the extracted parent ions (**1**, *m/z* 899) from plot (**a**); plot (**c**) corresponds to the 100-fold magnified TIC from the MS^2 of the selected parent ions (**1**, *m/z* 899); plot (**d**) corresponds to the 100-fold magnified TIC from the MS^4 of the selected aglycon ions (**4**, *m/z* 575); plot (**e**) corresponds to the 2000-fold magnified TIC of the sum of extracted ions, from plot (**d**), at *m/z* 303, 345 and 439; plot (**f**) corresponds to the 200-fold magnified TIC of the sum of extracted ions, from plot (**d**), at *m/z* 287, 329 and 423; and plot (**g**) corresponds to the 2000-fold magnified TIC for the sum of extracted ions, from plot (**d**), at *m/z* 271, 313, and 407. As can be seen, plot (**c**), corresponding to the TIC from the MS^2 of the selected isotopic parent ions (**1**, *m/z* 899), shows two major peaks and several minor ones. Evidently, not all the ions at *m/z* 899 must necessarily correspond to true "parent ions" from flavanol−anthocyanin adducts, since some of them could be generated from other components or simply be isotopic ions from much more intense ions at *m/z* 897/898, and so they would correspond to different structures. Consequently, all the peaks in plot (**c**) were checked for fragmentation and only a limited number of them gave an "expectable" mass spectrum. Interestingly, all the "expectable" mass spectra obtained were practically coincident and contained only two noticeable daughter ions at *m/z* 737 (**2** + **3**, M^+ − 162, 100%) and *m/z* 575 (**4**, M^+ − 162 − 162, ≈50%). Therefore, the putative flavanol−anthocyanin adducts sharing this molecular weight must be disubstituted (most probably at the 3 and 5 positions on the anthocyanidin) with hexoses (most probably with glucoses) and thus the remaining presumed flavanol−anthocyanin adducts, which do not fulfill this constraint, could be disregarded. It must be noted that in **Figure 2**, with the intent to limit its intricacy, neither the plot corresponding to the TIC from the MS^3 of the selected first isotopic daughter ions at *m/z* 737 (**2** + **3**, M^+ − 162), which yield the aglycon ion at *m/z* 575 (**4**, M^+ − 162−162), nor the plot corresponding to the TIC for the extraction of this latter ion are shown, but they were all in all similar to plot (**d**). Before the final assignation of the peaks in plot (**d**) to the flavanol−anthocyanin aglycons by direct inspection of their mass spectrum, essential preliminary information could be gained by means of the extraction, from plot (**d**), of the three characteristic

ions corresponding to each anthocyanidin. Hence, plot (**e**), which corresponds to the TIC of the sum of the extracted ions at *m*/*z* 303 (**6**, delphinidin), 345 (**7**, delphinidin + 42) and 439 (**8**, delphinidin + 136), strongly suggests the presence of the afzelechin−delphinidin and epiafzelechin−delphinidin aglycons at retentions times 18.70 and 24.86 min, or vice versa. Similarly, plots (**f**) and (**g**), which correspond to the TICs of the sum of the extracted ions at *m*/*z* 287 (**6**, cyanidin), 329 (**7**, cyanidin + 42) and 423 (**8**, cyanidin + 136), and at *m*/*z* 271 (**6**, pelargonidin), 313 (**7**, pelargonidin + 42) and 407 (**8**, pelargonidin + 136), respectively, suggest the presence of the aglycons catechin−cyanidin and epicatechin−cyanidin (at retention times 11.77 and 22.85 min, or vice versa) and gallocatechin−pelargonidin and epigallocatechin−pelargonidin (at retention times 14.18 and 19.13 min, or vice versa). Final confirmation was obtained by inspection of the mass spectra. Peaks at retention times 18.70 and 24.86 min gave the same mass spectrum, shown in **Figure 3A**, which confirms the presence of the afzelechin−delphinidin and epiafzelechin−delphinidin aglycons. Similarly, peaks at retention times 11.77 and 22.85 min gave the same mass spectrum, shown in **Figure 3B**, which confirms the presence of the catechin−cyanidin and epicatechin−cyanidin aglycons, and peaks at retention times 14.18 and 19.13 min gave the same mass spectrum, shown in **Figure 3C**, thus confirming the presence of the gallocatechin−pelargonidin and epigallocatechin−pelargonidin aglycons. **Figure 4** summarizes the results obtained with the 3,5-dihexoside derivatives of the flavanol−anthocyanin adducts, where plot (**a**) corresponds to the TIC from the MS of the sample; and plots (**b**), (**c**), (**d**), (**e**) and (**f**) correspond to the TICs from the MS^4 of the selected ions at *m*/*z* 607, 591, 575, 559 and 543, respectively, which in their turn are the aglycons of the parent ions at *m*/*z* 931, 915, 899, 883 and 867, respectively. Peaks have been numbered according to their retention times, but taking also into account the retention times of the additional flavanol−anthocyanin adducts detected in this study. More detailed information concerning these disubstituted adducts is given in **Table 2**, where 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.

Flavanol−Anthocyanin-3-hexosides. The operating steps carried out with the 3-hexoside derivatives of the flavanol−anthocyanin adducts were, in practice, identical to those with the 3,5-dihexoside derivatives. Nevertheless, let us considerer here a simpler case, that is, the parent ion at *m*/*z* 769 which, as indicated previously, would correspond only to the adducts (epi)gallocatechin−delphinidin-3-hexoside. The reason for this choice is that in this particular case the possible coelutions and interferences within the peaks from different isotopic flavanol−anthocyanin aglycons are avoided. In **Figure 5**, plot (**a**) corresponds to the TIC from the MS of the sample; plot (**b**) corresponds to the 50-fold magnified TIC for the extracted parent ion (**1**, R_6 = H, *m*/*z* 769) from plot (**a**); plot (**c**) corresponds to the 50-fold magnified TIC from the MS^2 of the selected parent ion at *m*/*z* 769 (**1**), which yields the aglycon ion (**4**, *m*/*z* 607); and plot (**d**) corresponds to the 50-fold magnified TIC from the MS^3 of the selected aglycon ion at *m*/*z* 607 (**4**, $M^+ - 162$). As usual, all the peaks in the TIC from the MS^2 of the selected parent ion [plot (**c**)] were checked for fragmentation, and only six peaks, which are marked with arrows, at retention times 13.57, 14.94, 16.08, 16.52, 18.50, and 24.72 min, gave a consistent fragmentation, that is, a mass spectrum containing a single ion at *m*/*z* 607 (**4**, $M^+ - 162$, the aglycon ion). As expected, these same peaks were also found in plot (**d**). Interestingly, whereas the mass spectrum from the peaks at retention times 13.57, 14.94, 16.08, and 18.50 min was practically the same, as shown in **Figure 6** and confirming the presence of the (epi)gallocatechin−delphinidin aglycons, the

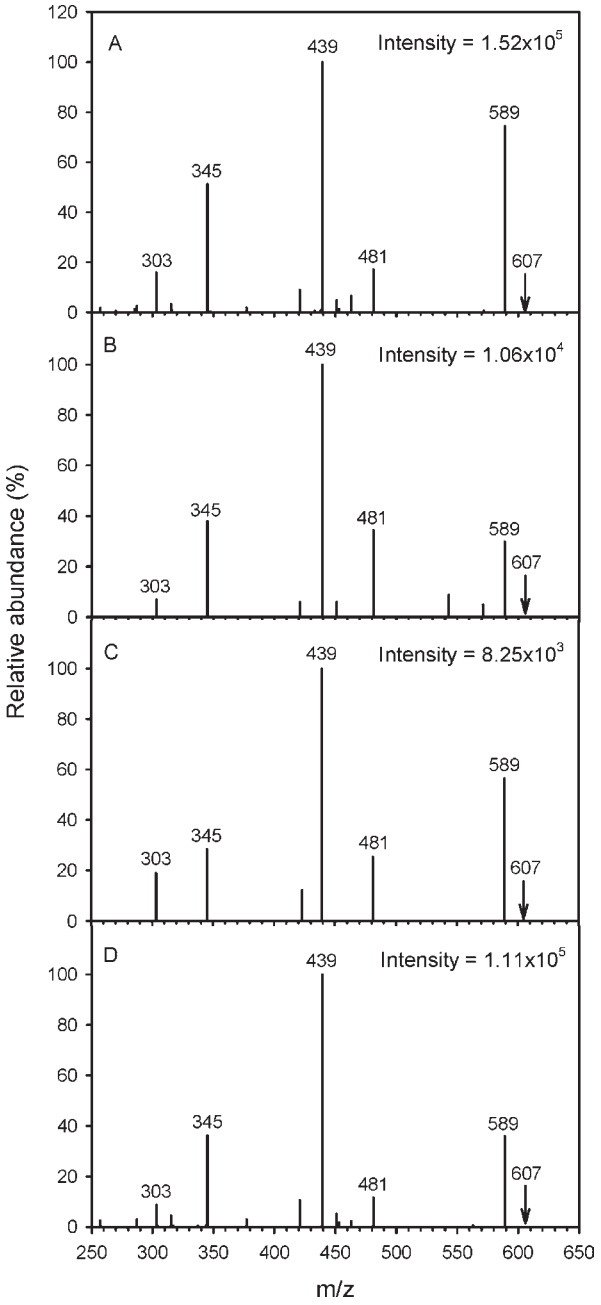

Figure 6. Mass spectra and total ion intensities from the peaks in the TIC from the MS^3 of the selected aglycon ion at *m*/*z* 607, with retention times (min): 13.57 (**A**), 14.94 (**B**), 16.08 (**C**), and 18.50 (**D**).

mass spectrum from the peaks at 16.52 and 24.72 min was also practically the same, but clearly different from the previous ones. Salas et al. (*29*) found that the reaction between malvidin-3-*O*-glucoside, in its hydrated form, and catechin leads to the formation of two flavanol−anthocyanin dimers. The major one was isolated and demonstrated to be catechin-(4α→8)-malvidin-3-*O*-glucoside, and therefore, the other should most probably be catechin-(4*β*→8)-malvidin-3-*O*-glucoside. Hence, the appearance of four peaks giving the same mass spectrum, which in addition were also detected in some other of the assayed molecular weights belonging to both mono- and disubstituted adducts, strongly suggests that the four possible 4→8 linked structural isomers of the detected flavanol−anthocyanin adducts, namely, flavanol-(4α/*β*→8)-anthocyanin and epiflavanol-(4α/*β*→8)-anthocyanin, could be present in pomegranate juice. Moreover, in all cases was observed a great difference in ion intensity (concentration)

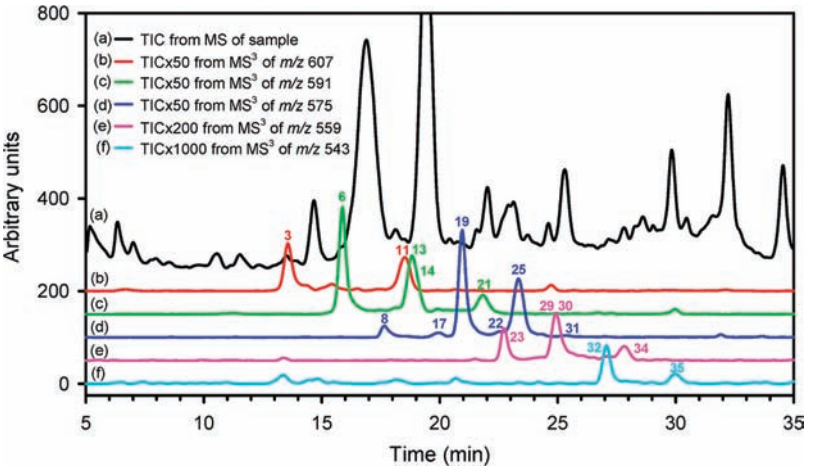

Figure 7. Graphical representation of the TIC from the MS of pomegranate juice, and the TICs from the MS^3 of the selected aglycon ions at *m/z* 607, 591, 575, 559 and 543, corresponding to the flavanol−anthocyanin-3-hexoside adducts with molecular weights at *m/z* 769, 753, 737, 721 and 705, respectively. For additional details about numbered peaks, see **Table 2**.

between two peaks, which were major ones, and the other two, which eluted between the two majors and whose ion intensity was clearly smaller. This difference in ion intensities, as well as the observed relative retention times, could probably by explained as a function of the steric hindrance of the flavanol−anthocyanin adduct, that is, the ability of a given anthocyanin to enter on the C4 position at axial or equatorial position depending on the structure of the target molecule, flavanol or epiflavanol (*23*, *29*). In any way, the concentration of these two minor peaks was always so small that the authors could not completely disregard, at least in some cases, a fortuitous combination of factors given the expected ions and pattern. As a consequence, these minor peaks have not been included in this work as detected flavanol−anthocyanin adducts, although their genesis and mass spectra fulfill all the requirements. **Figure 7** summarizes the results with the 3-hexoside derivatives of the flavanol−anthocyanin adducts, where plot (**a**) corresponds to the TIC from the MS of the sample; and plots (**b**), (**c**), (**d**), (**e**) and (**f**) correspond to the TICs from the MS^3 of the selected ions at *m/z* 607, 591, 575, 559 and 543, respectively, which in their turn are the aglycons of the parentions at *m/z* 769, 753, 737, 721 and 705, respectively. Peaks are numbered according to their retention times, but taking also into account the retention times of the already detected flavanol−anthocyanin adducts. More detailed information about these components is given in **Table 2** where, as indicated previously, 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.

Concerning the peaks at retention times 16.52 and 24.72 min, their mass spectrum contained only two noticeable ions, one at *m/z* 589 (≈35%) and the other at *m/z* 439 (100%). Since these two daughter ions are also present in the mass spectrum of the (epi)gallocatechin−delphinidin aglycon, their genesis could most probably be the same, that is, the ion at *m/z* 589 ($M^+ - 18$) would come from a loss of water and the ion at *m/z* 439 ($M^+ - 168$) would come from a retro-Diels−Alder fragmentation of the C ring of an (epi)gallocatechin. In short, this simple fragmentation pathway, in conjunction with the absence of any other relevant fragmentation, strongly indicates a structure compatible with those of the delphinidin−(epi)gallocatechin aglycons, that is, the other type of adducts derived from the direct condensation between flavanols and anthocyanins (*26*), and where the anthocyanin occupies the upper part of the dimer. In any case, this study is in progress and the results will be published elsewhere.

The results of this work demonstrate that the detection and identification of direct condensation flavanol−anthocyanin adducts, even in very minor concentration, can be a rather straightforward task, provided that the necessary instrumentation is available and that a focused and systematic methodology is followed.

The relatively great number of direct condensation flavanol−anthocyanin adducts detected in this fresh pomegranate juice is in good accordance with the high reactivity of phenolic compounds and with the fact that most of the reactions within them start as soon as the plant cells are damaged or broken and continues throughout the processing and storage, thus adding more and more complexity to the sample (*12*). Hence, it seems reasonable to assume that most of the plant-based foods containing anthocyanins should contain also the corresponding flavanol−anthocyanin adducts in more or less concentration. This could have important food technological implications due to the effects of the appearance of anthocyanin-derived adducts on the original color and taste of the fresh food (*12*). These effects have been extensively studied in red wines during their maturation and aging, but in this alcoholic beverage the direct condensation between flavanols and anthocyanins to yield flavanol−anthocyanin adducts is of relatively minor importance due to the presence of several yeast metabolites, such as acetaldehyde, vinylphenol, pyruvic acid, etc., which strongly induce alternative reactions such as the formation of ethyl-linked flavanol−anthocyanin adducts and pyranoanthocyanin derivatives, among others (*30*). In nonfermented plant-based foods, on the contrary, particularly if they are rich in anthocyanins and flavan-3-ols (or tannins) and have to be stored for some time, the direct condensation between flavanols and anthocyanins could be the main pathway for the formation of anthocyanin adducts. Hence, in these particular cases the detection and identification of these adducts, as well as the monitoring of their time evolution during storage, could be important for quality control and, perhaps, could also aid to explain some product modifications not yet well-understood.

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