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On line characterization of 58 phenolic compounds in *Citrus* fruit juices from Spanish cultivars by high-performance liquid chromatography with photodiode-array detection coupled to electrospray ionization triple quadrupole mass spectrometry

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ABSTRACT

Polyphenol profile of *Citrus* juices of sweet orange, tangerine, lemon and grapefruit from Spanish cultivars was obtained by High-Performance Liquid Chromatography with Diode Array Detection coupled to Electrospray ionization and Triple Quadrupole Mass Spectrometry. Fifty eight phenolic compounds of five different classes were identified in these *Citrus* juices. Flavanone: *O*-dihexoside of naringenin; flavones: apigenin-7-*O*-rutinoside-4′-*O*-glucoside, luteolin-7-*O*-neohesperidoside-4′-*O*-glucoside, luteolin-6-*C*-glucoside, 6,8-di-*C*-acylhexosides of chrysoeriol and diosmetin, 6*C*- and 8*C*-glucoside-*O*-pentoside of apigenin, apigenin-6-*C*-hexoside-*O*-hexoside and apigenin-8-*C*-hexoside-*O*-acylrhamnoside; flavonols: 7-*O*-rutinosides of quercetin, kaempferol, isorhamnetin and tamarixetin, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside-7-*O*-glucoside, tamarixetin-3-*O*-rutinoside-7-*O*-glucoside, isorhamnetin-3-*O*-hexoside-7-*O*-rhamnosylhexoside, 3-*O*-rhamnoside-7-*O*-rhamnosylhexoside of quercetin and isorhamnetin and kaempferol-3-*O*-rhamnosylhexoside-7-*O*-rhamnoside; hydroxycinnamic acids: *O*-hexoside of ferulic and sinapic acid; and, coumarins: *O*-hexoside and *O*-rhamnosylhexoside of scopoletin, had not previously been reported in *Citrus* juices to our knowledge. Structures have been assigned on the basis of the complementary information obtained from retention time, UV–visible spectra, scan mode MS spectra, and fragmentation patterns in MS^2 spectra obtained using different collision energies. A structure diagnosis scheme is provided for the identification of different phenolic compounds.

1. Introduction

The positive effects of *Citrus* fruit consumption on human health were of common knowledge centuries before researchers begun to unravel the complexity of such food matrices. Over the past decades, a large number of studies have been carried out with the aim of identifying the bioactive components present in different parts of *Citrus* fruits, in an attempt to gain a deeper understanding of the correlation between diet, health benefits and reduced risk of diseases.

Nowadays, an amount of data has been collected on the biomedical properties of many relevant nutraceuticals [1]. In this context, several epidemiological studies have associated the consumption of phenolic compounds, and more specifically flavonoids, with lower risks of different types of cancer [2] and cardiovascular diseases [3], and have shown that they posses antioxidant, anti-inflammatory and anti-ageing activity [4]. *Citrus* fruits are the main winter fruits consumed in the Mediterranean diet, so they are the main source of dietary flavonoids, especially flavanone and flavones with flavonols present in lower concentration [5] although polymethoxylated flavones have been also found in large amounts in the peel of some *Citrus* [6]. Flavonoids found in different parts of *Citrus* fruits usually do not occur normally as aglycones [7,8] but rather as glycosides [9].

Apart from their beneficial properties in food, which have conferred on them a relevant role as nutraceuticals [10], polyphenols are chemotaxonomic markers due to their specifity and ubiquity, and they have proven to be chemical markers for food authentication demanded by food producers, consumers and regulatory bodies [11–13]. Characteristic phenolic compounds

Abbreviations: Nar, Naringenin; Eri, Eriodictyol; Isk, isosakuranetin; Hes, hesperetin; Heri, homoeriodictyol; Lut, luteolin; Dio, diosmetin; Chrys, chriysoeriol; Api, apigenin; Kaem, kaempferol; Que, quercetin; Iso, isorhamnetin; Tam, tamarixetin; Fer, ferulic acid; Snp, sinapic acid; Sco, scopoletin; rha, rhamnoside; hex, hexoside; pent, pentoside; glc, glucoside; rut, rutinoside; nhes, neohesperidoside; Acylgly, acylglycoside; FVNN, flavanone; FVN, flavone; FVL, flavonol; DFVL, Dihydroxyflavonol; HCA, hydroxycinnamic acid; CM, coumarin

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have been successfully used for the determination of adulteration of *Citrus* juices [14–16] and *Citrus* jam [17] with cheaper fruits.

For the investigation of structure–activity relationships and food quality control of natural polyphenolic compounds, it is also important to have access to rapid and reliable methods for the analysis and identification of these natural phenolic compounds in all their many forms. Among the methods used for the determination of phenolic compounds, the most widely used are based on reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to diode array detection (DAD) and mass spectrometry (MS) with atmospheric pressure ionization techniques, i.e., electrospray ionization (ESI) or atmospheric pressure chemical ionization (APcI). With the use of tandem MS technologies (MS/MS) in combination with collision-induced dissociation (CID), MS/MS spectra of a range of flavonoid structures have been investigated and compared, obtaining fragmentation rules and fragmentation patterns that enable discrimination and identification of a wide range of phenolic compounds [18–20].

In the present paper, a comprehensive characterization of phenolic compounds in *Citrus* juices (sweet orange, tangerine, lemon and grapefruit) from Spanish cultivars by HPLC-DAD-ESI-CID-MS/MS is reported. The structural information provided by online technical HPLC-DAD-ESI-CID-MS/MS scan and product ion scan mode led to identify and characterize successfully 58 phenolic compounds in *Citrus* fruit juices using the mechanisms and fragmentation patterns established in the previous study with phenolic compounds standards [19]. Although some of the phenolic compounds have been previously described in literature, 25 phenolic compounds have been detected for the first time in *Citrus* in this work.

2. Experimental

2.1. Reagents, solvents and standard phenolics

Methanol and dimethyl sulfoxide (Romil, Chemical Ltd, Heidelberg, Germany) were of HPLC grade. Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). Glacial acetic acid, ascorbic acid and sodium fluoride provided by Merck (Darmstadt, Germany) were of analytical quality. All solvents used were previously filtered through 0.45 μm nylon membranes (Lida, Kenosha, WI, USA).

Phenolics standards were supplied as follows: eriodictyol-7-*O*-rutinoside, eriodictyol-7-*O*-neohesperidoside, naringenin-7-*O*-rutinoside, hesperetin-7-*O*-rutinoside, hesperetin-7-*O*-neohesperidoside, isosakuranetin-7-*O*-rutinoside, hesperetin, homoeriodictyol, ferulic acid, sinapic acid, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucofuranoside, quercetin-3-*O*-glucopyranoside, quercetin-3-*O*-rhamnoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, kaempferol-7-*O*-neohesperidoside, kaempferol-3-*O*-robinoside-7-*O*-rhamnoside, isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, isorhamnetin, tamarixetin, myricetin, scopoletin, luteolin-7-*O*-glucoside, luteolin-6-*C*-glucoside, luteolin-8-*C*-glucoside, luteolin-3′,7-di-*O*-glucoside, luteolin-4′-*O*-glucoside, diosmetin-7-*O*-rutinoside, apigenin-7-*O*-glucoside, apigenin-6-*C*-glucoside, apigenin-8-*C*-glucoside, apigenin-7-*O*-neohesperidoside, apigenin-7-*O*-rutenoside, diosmetin, chrysoeriol and sinensetin from Extrasynthèse (Genay, France); while naringenin, 5′-caffeoylquinic acid, caffeic acid, *p*-coumaric acid and quercetin-3-*O*-rutinoside were provided by Sigma-Aldrich Chemie (Steinheim, Germany); apigenin-8-*C*-glucoside-4′-*O*-rhamnoside, kaempferol-3-*O*-(*p*-coumaroyl)glucoside, tangeretin and nobiletin by Chromadex (Santa Ana, CA, USA); and naringenin-7-*O*-neohesperidoside, quercetin dehydrated and apigenin by Fluka Chemie (Steinheim, Germany).

All stock standard solutions (in concentrations ranging from 250 to 2500 μg/mL, depending on each phenolic compound) were prepared in methanol, except for hesperetin-7-*O*-rutinoside, hesperetin, homoeriodictyol, chrysoeriol and isorhamnetin that was dissolved with water–dimethyl sulfoxide (80:20, v/v), and all were stored at 4 °C in darkness.

2.2. Fruit samples

Fruits of four different *Citrus* species: sweet orange (*Citrus sinensis*) (nine cultivars), tangerine (*Citrus reticulate* and *Citrus unshiu*) (seven cultivars), lemon (*Citrus lemon)* (four cultivars) and grapefruit (*Citrus paradise)* (five cultivars), produced in Spain during the years 2003–2005 were purchased from a local market at maturity.

2.3. Citrus juice preparation

Three batches of fruit (1 kg) were constituted for each fruit cultivar and harvest. Each batch was peeled separating the flavedo and the albedo from the pulp and squeezed using a home juicer. The collected juice after measuring its volume, was mixed with 50 mL of an aqueous solution containing ascorbic acid 0.2 g/mL and sodium fluoride 0.2 g/mL, in order to inactive polyphenoloxidases and prevent phenolic degradation [21], and centrifuged at 6000 r.p.m. for 15 min at 4 °C. Aliquots of 1 mL were sampled, stored at −20 °C and lyophilized later. The freeze-dried material was stored at room temperature in a desiccator in darkness until analysis.

2.4. Analytical procedure

2.4.1. Solvent extraction of freeze-dried samples and RP-HPLC

Extraction was performed following a previously optimized procedure [22]. The HPLC system was a Waters (Milford, USA) Alliance 2695 coupled to a Waters 2996 DAD. A reversed-phase Phenomenex (Torrance, USA) Luna C18(2) column (150 × 4.6 mm i.d. and particle size 3 μm) with a Waters Nova-Pack C18 guard column (10 × 3.9 mm i.d., 4 μm) was used. A gradient program was employed [22].

2.4.2. Mass spectrometry

Mass spectra were obtained on a Micromass (Milford, MA, USA) Quattro micro-triple quadrupole mass spectrometer coupled to the exit of the diode array detector and equipped with a Z-spray ESI source. A flow of 70 μL/min from the DAD eluent was directed to the ESI interface using a flow-splitter. Nitrogen was used as desolvation gas, at 300 °C and a flow rate of 450 L/h, and no cone gas was used. A potential of 3.2 kV was used on the capillary for positive ion mode and 2.6 kV for negative ion mode. The source block temperature was held at 120 °C.

Two independent runs, one for the MS^1 full scan mode and another for MS^2 product ion scan mode were carried out at 1 scans/s and inter-scan delay of 0.1 s. MS^1 full scan spectra, within the *m*/*z* range 50–1000, were performed in the positive mode at different cone voltages (15, 30 and 45 V) and in the negative mode at −30 V. MS^2 product ion spectra in positive mode were recorded using argon as collision gas at 1.5×10^{-3} mbar and under different collision energies in the range 5–40 eV and optimized cone voltages. The optimum cone voltages were those which produced the maximum intensity for protonated molecular ion $[M+H]^+$ and protonated aglycone ion $[Y_0]^+$ in the previous MS^1 experiments.

The nomenclature adopted to denote the fragment ions for glycoconjugates was proposed by Domon and Costello [23] (Fig. 1). The flavonoid aglycone fragment ions have been designed according to the nomenclature proposed by Ma et al. [24] (Fig. 2).

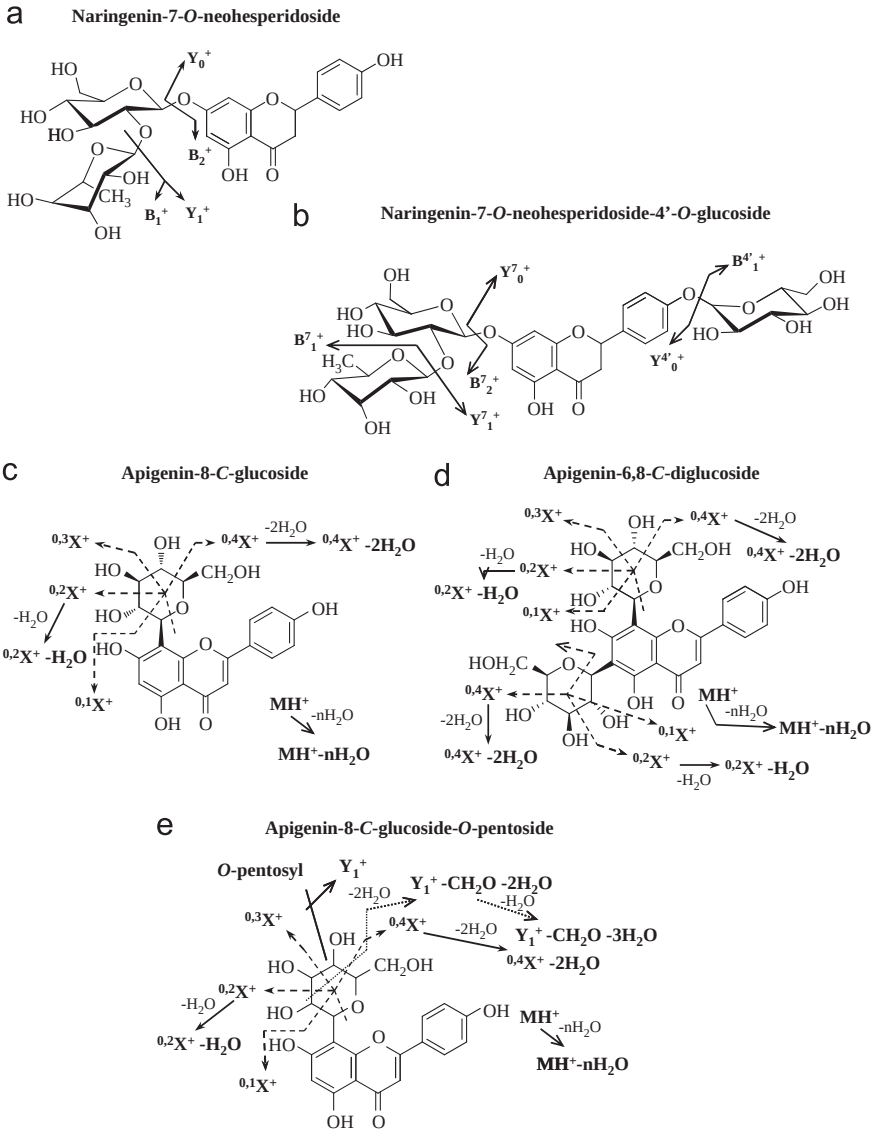


Fig. 1. Main fragmentation observed for (A) protonated flavonoid-*O*-diglycosides; (B) protanted flavonoid-*O*-triglycosides; (C) protonated flavonoid-*C*-monoglycoside; (D) protonated flavonoid-di-*C*-glycoside; and (E) protonated flavonoid-*O*,*C*-glycoside in tandem mass spectrometry.

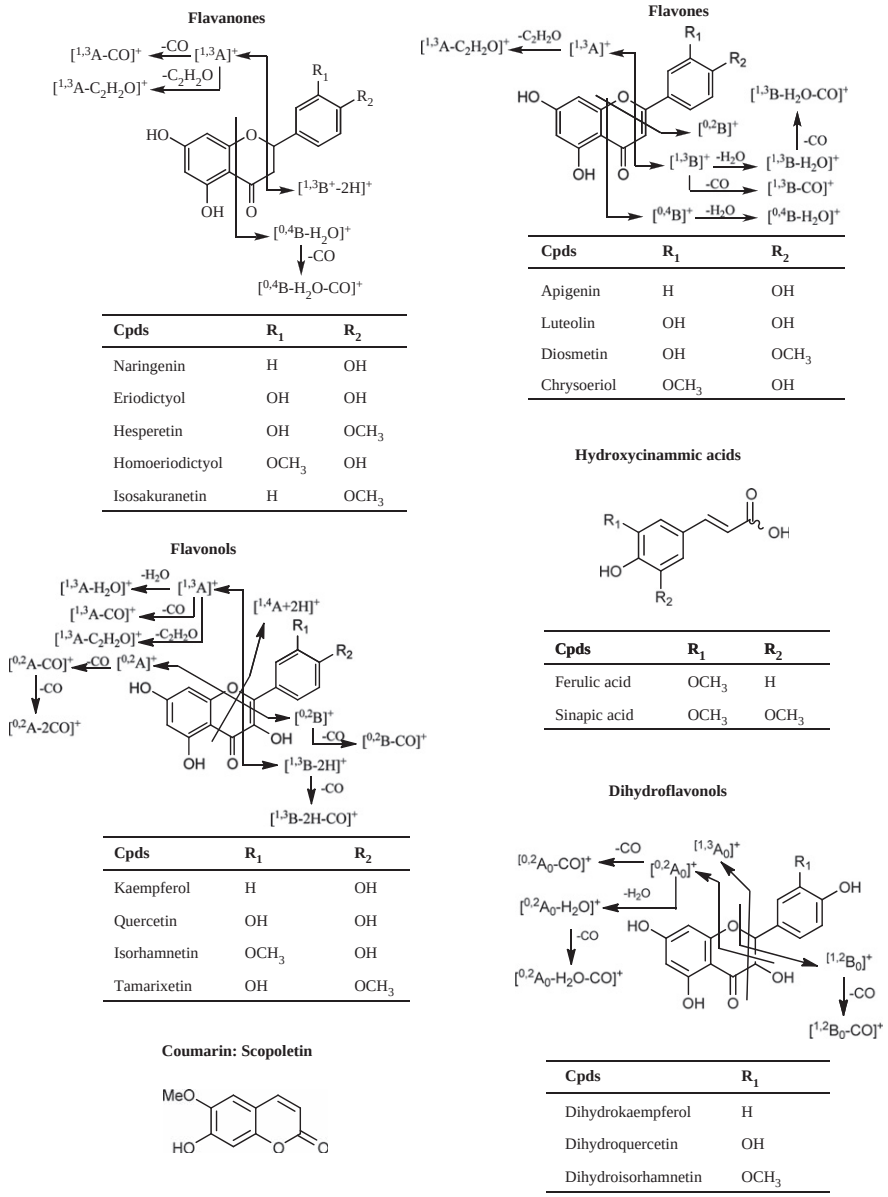
2.4.3. Identification and quantitation of phenolic compounds

The identification of the phenolic compounds for which standards were available was carried out by the comparison of their retention time, their UV–visible spectra and ESI-MS/MS spectra recorded in MS^1 full scan in positive and negative mode and MS^2 product ion mode using as precursor ion the protonated molecule $[M+H]^+$ and the protonated aglycone $[Y_0]^+$ with those obtained by injecting standards in the same conditions, while the identity of other compounds was elucidated using the UV–vis spectrum to assign the phenolic class [19,25], the MS^1 full scan in positive and negative mode to determine the molecular weight, the MS^2 product ion spectrum using the $[M+H]^+$ ion as precursor to assign the protonated aglycone $[Y_0]^+$ and fragmentations observed in both MS^2 product ion spectra using $[M+H]^+$ or $[Y_0]^+$ as precursors to elucidate other structural details. Additionally, the chromatographic elution order aided in some structural assignments as it was previously described [19].

Quantitation was performed using integration areas in the calibration regression of the standards most similar to each phenolic compound quantified. Thus, flavanones and dihydroflavonols were quantified as naringenin-7-*O*-rutinoside; apigenin glycosides as apigenin-7-*O*-glucoside; luteolin, diosmetin, and chrysoeriol glycosides as luteolin-7-*O*-glucoside; quercetin and kaempferol glycosides as quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside, respectively; isorhamnetin and tamarixetin glycosides as isorhamnetin-3-*O*-rutinoside; ferulic and sinapic acid derivates as 5′-caffeoylquinic and sinapic acid, respectively; and scopoletin glycosides as scopoletin. These concentrations were corrected with the recovery factors previously published [22].

3. Results and discussion

The combination of both ionization modes (positive and negative) in MS^1 full scan mode gave extra certainly to the molecular mass determination. The negative ion mode provides the highest sensitivity and results in limited fragmentation, making it most suited to infer the molecular mass of the separated flavonoids, especially in cases where concentration is low [18]. In addition, because only the quasi-molecular ions are able to form adducts, clusters and/or molecular complexes with mobile phase species in the electrospray ionization source, their presence in the MS spectra was very useful to carry out the unequivocal identification of the $[M+H]^+$ or



Cpds	R_1	R_2
Naringenin	H	OH
Eriodictyol	OH	OH
Hesperetin	OH	OCH_3
Homoeriodictyol	OCH_3	OH
Isosakuranetin	H	OCH_3

Cpds	R_1	R_2
Apigenin	H	OH
Luteolin	OH	OH
Diosmetin	OH	OCH_3
Chrysoeriol	OCH_3	OH

Cpds	R_1	R_2
Kaempferol	H	OH
Quercetin	OH	OH
Isorhamnetin	OCH_3	OH
Tamarixetin	OH	OCH_3

Cpds	R_1	R_2
Ferulic acid	OCH_3	H
Sinapic acid	OCH_3	OCH_3

Cpds	R_1
Dihydrokaempferol	H
Dihydroquercetin	OH
Dihydroisorhamnetin	OCH_3

Fig. 2. Structures of polyphenols and fragmentation pathways of the aglycones studied in *Citrus* fruit juices.

$[M-H]^-$ ion and hence determining the molecular weight of the unknown compounds. In this sense, the sodium adduct $[M+Na]^+$ at 22 u above the proposed protonated molecular ion in positive mode and intense adducts with HSO_4^- and AcO^- from the mobile phase in negative mode were of a great relevance.

The study of ESI(+)-MS/MS product ion spectra, obtained using as precursor the ion the protonated molecule $[M+H]^+$ and the voltage cone previously optimized in MS experiments, provided the fragmentation pathways of the different classes of flavonoids glycoconjugates present in Citrus juices. The structural information obtained with regard to structure characterization was (1) the type of carbohydrates (mono-, di- or trisaccharides), (2) the sequence of the glycan part, (3) interglycosidic linkages and (4) the aglycone moiety.

In addition, the study of ESI(+)-MS/MS product ion spectra, obtained using as precursor ion the protonated aglycone $[Y_0]^+$, showed the fragmentation pathways of the different classes of flavonoids aglycones. In this case, an unique compromise value of 35 eV for the collision energy was used for all aglycones, allowing the observation of $^{i,j}A^+$ and $^{i,j}B^+$ ions which require cleavage of two bonds of the C-ring and are the most useful fragmentations in terms of flavonoid aglycone identification. These ions, most of which can be rationalized by retro-Diels–Alder (RDA) reactions, are the most diagnostic fragments for flavonoid identification since they provide information on the number and type of substituents in the A- and B-rings [18,19].

Structures and main fragmentation pathways of each class of phenolic compounds studied are presented in Figs. 1 and 2. The 58 polyphenolic compounds shown in Table 1 belong to different phenolic families: flavanones, flavones, flavonols, dihydroflavonols, hydroxycinnamic acids and coumarins. The total concentration of phenolic compounds in the four species of Citrus fruit juices studied was 548–1407 mg/L for sweet orange juices; 215–1335 mg/L for tangerine juices; 658–1538 mg/L for lemon juices; 1173–2216 mg/L for grapefruit juices.

Other polyphenolic classes, such as anthocyanins, flavan-3-ols, dihydrochalcones, hydroquinones and hydroxybenzoic acids,

Table 1
Polyphenolic composition in *Citrus* fruit juices from Spanish cultivars.

Comp.no.[c,a]	Class[b]	Polyphenol	RT (min)	Or	Ta	Le	Gr	Ref.
1	CM	Scopoletin-*O*-hexoside	20.5				++	
2	DFVL	Dihydroquercetin-7-*O*-rutinoside	21.2		+			[26]
3	CM	Scopoletin-*O*-rhamnosylhexoside	24.9				+	
4	HCA	O-hexoside of ferulic acid	25.2	+++	+++	++	+++	
5	DFVL	Dihydrokaempferol-7-*O*-rutinoside	27.1		+		+	[26]
6	HCA	*O*-hexoside of sinapic acid	27.3	++	++	++	+	
7	FVL	Quercetin-3-*O*-rutinoside-7-*O*-glucoside	33.7	++	++	++		[27,28]
8	DFVL	Dihydroisorhamnetin-7-*O*-rutinoside	36.2	+	+	+	+	[26]
9	FVN	Luteolin-6,8-di-*C*-glucoside	37.8	++		++		[5,29–32]
10	FVNN	Naringenin-7-*O*-rutinoside-4′-*O*-glucoside	47.1	+++	+++		+++	[31,33–35]
11	FVNN	Eriodictyol-7-*O*-rutinoside-4′-*O*-glucoside	47.6			+++		[28]
12	FVN	Apigenin-6,8-di-*C*-glucoside	48.0	+++	+++	+++	+++	[5,27,36–41]
13	FVL	Kaempferol-3-*O*-rutinoside-7-*O*-glucoside	49.4	+	+			[28]
14	FVNN	Naringenin-7-*O*-neohesperidoside-4′-*O*-glucoside	53.2				+++	[35,42,43]
15	FVL	Isorhamnetin-3-*O*-hexoside-7-*O*-rhamnosylhexoside	53.6	++	+			
16	FVL	Isorhamnetin-3-*O*-rutinoside-7-*O*-glucoside	54.3	++	+	++		
17	FVN	Chrysoeriol-6,8-di-*C*-glucoside	55.4			++		[27]
18	FVN	Apigenin-7-*O*-rutinoside-4′-*O*-glucoside	56.3			++		
19	FVL	Tamarixetin-3-*O*-rutinoside-7-*O*-glucoside	56.8		+			
20	FVN	Diosmetin-6,8-di-*C*-glucoside	58.1	+	++	+++		[5,27,29,36,38–40,44]
21	FVN	Luteolin-7-*O*-neohesperidoside-4′-*O*-glucoside	58.2				+	
22[a]	FVNN	Eriodictyol-7-*O*-rutinoside	62.5	++	++	++++		[5,45–47]
23[a]	FVN	Luteolin-6-*C*-glucoside	63.8			+		
24	FVNN	Hesperetin-7-*O*-rutinoside-3′-*O*-glucoside	64.0	+	+			[48]
25	FVNN	Naringenin-*O*-hexosylhexoside	66.9				++	
26	FVL	Quercetin-3-*O*-rhamnoside-7-*O*-rhamnosylhexoside	67.7	++	+			
27[a]	FVN	Apigenin-8-*C*-glucoside	68.6	+	+			[29,42,49]
28	FVN	Chrysoeriol-6,8-di-*C*-hexosideacylhexoside	71.5			++		
29	FVL	Quercetin-7-*O*-rutinoside	72.2	++	++	++	++	
30	FVN	Diosmetin-6,8-di-*C*-hexosideacylhexoside	72.8			++		
31	FVN	Apigenin-8-*C*-glucoside-*O*-pentoside	73.6	++		+	+	
32	FVN	Apigenin-6-*C*-hexoside-*O*-hexoside	74.7				++	
33	FVN	Apigenin-6-*C*-glucoside-*O*-pentoside	75.1	++		++	++	
34[a]	FVNN	Naringenin-7-*O*-rutinoside	77.6	++++	+++	++	++++	[40,50–54]
35	FVN	Diosmetin-8-*C*-glucoside	79.1		+	++		[42,55]
36	FVL	Kaempferol-3-*O*-rhamnosylhexoside-7-*O*-rhamnoside	79.1	+	++			
37	FVN	Luteolin-7-*O*-rutinoside	80.3		++	+++		[56,57]
38	FVL	Isorhamnetin-3-*O*-rhamnoside-7-*O*-rhamnosylhexoside	80.8	++	+			
39	FVNN	Homoeriodictyol-7-*O*-rutinoside	81.3			+		[27]
40[a]	FVNN	Naringenin-7-*O*-neohesperidoside	82.8				++++	[40,50–53,58–61]
41[a]	FVL	Quercetin-3-*O*-rutinoside	83.2	++	++	+++		[29,40,58,62,63]
42	FVN	Diosmetin-6-*C*-glucoside	85.3		+	+++		[36,40,44]
43[a]	FVNN	Hesperetin-7-*O*-rutinoside	87.2	++++	++++	++++	+++	[40,50–54,58]
44	FVL	Kaempferol-7-*O*-rutinoside	88.7		+			
45	FVN	Apigenin-8-*C*-hexoside-*O*-acylrhamnoside	91.6	++			+	
46[a]	FVNN	Hesperetin-7-*O*-neohesperidoside	91.9				+++	[52,64]
47[a]	FVN	Apigenin-7-*O*-rutinoside	93.0		++	++	+	[42,65]
48	FVL	Isorhamnetin-7-*O*-rutinoside	93.0	++	+	++		
49	FVN	Chrysoeriol-7-*O*-rutinoside	95.5		++	++		[66]
50	FVL	Tamarixetin-7-*O*-rutinoside	96.2		+			
51[a]	FVN	Apigenin-7-*O*-neohesperidoside	98.6				+++	[29,35]
52[a]	FVN	Diosmetin-7-*O*-rutinoside	99.6		+	++		[5,29,36,41,67]
53[a]	FVL	Kaempferol-3-*O*-rutinoside	99.8	+	++		+	
54[a]	FVL	Isorhamnetin-3-*O*-rutinoside	104.8	++	++	++		[28]
55[a]	FVNN	Isosakuranetin-7-*O*-rutinoside	126.5	+++	+++	++	++	[5,54,56,58,68]
56	FVNN	Naringenin-*O*-rhamnosylmalonylhexoside-1	131.7				+++	[50,69,70]
57	FVNN	Naringenin-*O*-rhamnosylmalonylhexoside-2	133.0				+++	[50,69,70]
58	FVNN	Isosakuranetin-7-*O*-neohesperidoside	137.4				+++	[61,71]

+, concentration ≤ 1 μg/mL; ++, concentration from 1 to 10 μg/mL; +++, concentration from 10 to 100 μg/mL; ++++, concentration > 100 μg/mL.

[a] The phenolic compounds for which standard were available.

[b] Phenolic class: flavanones (FVNN), flavones (FVN), flavonols (FVL), dihydroflavonols (DFVL), hydroxycinnamic acids (HCA) and coumarins (CM).

were searched but not detected. As an example, Fig. 3 shows UV–vis and MS data for peak (No. **14**).

3.1. Flavanones

3.1.1. Flavanone-*O*-diglycosides

Eriodictyol-7-*O*-rutinoside (compound **22**), naringenin-7-*O*-rutinoside (compound **34**), naringenin-7-*O*-neohesperidoside (compound **40**), hesperetin-7-*O*-rutinoside (compound **43**), hesperetin-7-*O*-neohesperidoside (compound **46**) and isosakuranetin-7-*O*-rutinoside (compound **55**) standards allowed the unequivocal identification of these six flavanones in *Citrus* fruit juice extracts (Table 1).

These flavanones have been already characterized as components of *Citrus* fruit [56]. Thus, eriodictyol-7-*O*-rutinoside, naringenin-7-*O*-rutinoside and hesperetin-7-*O*-rutinoside were found in juice extracts of the four different species of *Citrus* genus

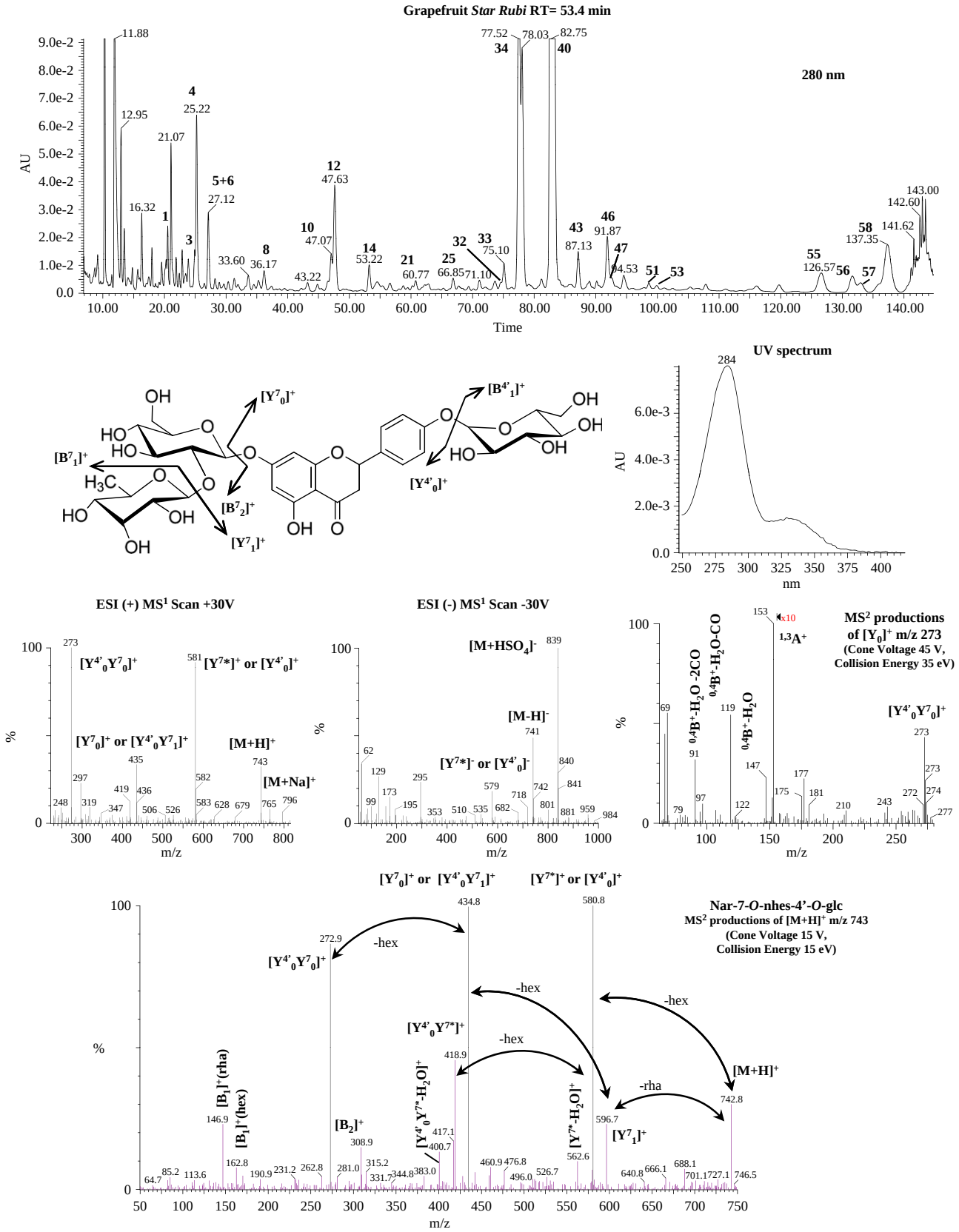


Fig. 3. LC-DAD-ESI-CID-MS/MS data used for peak assignment (data of peak 14).

studied according to bibliographical sources [5,40,45–47,50–54,58]. In this work, eriodictyol-7-*O*-rutinoside was not detected in grapefruit juices. The naringenin-7-*O*-neohesperidoside was only found in grapefruit juice extracts [58], unlike other authors who also pointed to the presence of the latter in sweet orange, tangerine and lemon juices [59]. The hesperetin-7-*O*-neohesperidoside was only

found in grapefruit juice extracts [52,64] while the isosakuranetín-7-*O*-rutinoside was detected in sweet orange, tangerine, lemon and grapefruit juice extracts according to Refs. [54,56,68].

Compound **58** (RT 137.4 min), present in the grapefruit juice extracts, was tentatively identified as isosakuranetin-7-*O*-neohesperidoside according to UV and MS/MS spectra and bibliographic sources [61,71] (Table 1). This compound showed the same UV spectra as flavanone standards. The protonated and deprotonated molecular ions detected in MS^1 scan spectra in positive and negative modes were 595 and 593, respectively. The ESI(+)-MS/MS product ion spectra obtained using as precursor ion the protonated aglycone $[Y_0]^+$ (m/z 287) revealed the characteristic fragmentation pattern of the isosakuranetin aglycone (Fig. 2, Table 2 in the Electronic Supplementary Material, ESM) and the ESI(+)-MS/MS product ion spectra obtained using as precursor ion the $[M+H]^+$ ion yielded the product ions $[Y_1]^+$, $[Y*]^+$ and $[Y_0]^+$ at m/z 449, 433 and 287 and $[B_1]^+$ and $[B_2]^+$ at m/z 147 and 309, respectively, characteristic of the glycan sequence (Fig. 1A, Table 2 in ESM). Differentiation between flavanone rutinosides and their isomeric neohesperidosides were carried out based on the relative intensities of $[Y^*]^+$ and $[Y_0]^+$. The relative intensities $[Y^*]^+ < [Y_0]^+$ point this compound as isosakuranetin-7-*O*-neohesperidosdide [72]. Moreover, these compound eluted at a higher retention time than its corresponding rutinoside isomer (compound **55**), in the same way as that of other neohesperidosides [72].

Compound **39** (R. T. 81.3 min), detected in lemon juice extracts, was tentatively identified as homoeriodictyol-7-*O*-rutinoside according to Ref. [27] (Table 2 in ESM). This compound also showed the same UV spectra as flavanone standards and a high intensity of ion at m/z 611 corresponding to $[M+H]^+$ ion in MS^1 scan spectra. The ESI(+)-MS/MS product ion spectra obtained using as precursor ion the $[M+H]^+$ ion yielded a similar fragmentation pattern as the hesperetin-7-*O*-rutinoside. This fact reveals that this compound is 1→6 *O*-rutinoside at C7 ($[Y^*]^+ \geq [Y_0]^+$). The aglycone identity was determined tentatively as homoeriodictyol due to its earlier elution in relation to its hesperetin isomer. This behavior has been confirmed with homoeriodictyol and hesperetin standards. The MS^2 product ion spectra of these protonated aglycones were practically identical and did not give extra data to distinguish between these aglycones.

According to UV and fragmentation pattern, compound **25** (RT 66.9 min), present in grapefruit juices extract, was tentatively characterized as naringenin-*O*-hexosylhexoside in *Citrus* juices for the first time in this work (Table 2 in ESM). The MS^1 scan spectra allowed to detect the protonated and deprotonated molecular ions at m/z 597 and 595 in positive and negative modes, respectively. The ESI(+)-MS/MS product ion spectra from the protonated aglycone ($[Y_0]^+$ at m/z 273) was the same as that for naringenin aglycone standard. ESI(+)-MS/MS product ion spectra from $[M+H]^+$ (m/z 597) showed two intense ions $[Y_1]^+$ and $[Y_0]^+$ at m/z 435 and 273, resulting from the loss of a residue of hexose ($[Y_1]^+$) and the disaccharide hexosylhexose ($[Y_0]^+$), and a weak ion at m/z 417, generated by the loss of one molecule of H_2O from the $[Y_1]^+$ ion. The lower mass area provided useful information to differentiate between di-*O*-glycosides and *O*-diglycosides. The detection of $[B_1]^+$ and $[B_2]^+$ ions at m/z 163 and 325, respectively, could predict this compound as an *O*-diglycoside composed of two hexose units.

3.1.2. Flavanone-O-acyldiglycoside

Compounds **56** (RT 131.7 min) and **57** (RT 133.0 min), detected in grapefruit juice extracts [50], were characterized as naringenin-*O*-rhamnosylmalonylhexoside-1 and -2 according to UV and fragmentation pattern. The MS^1 scan experiments and UV spectra led readily to the determination of these flavonoids as naringenin acylglycosides according to bibliographic data [69,70] (Table 2 in ESM). In addition to this, the presence of acylation explains their late elution. The MS^1 scan spectra in positive ion mode for these flavanones showed an intense peak at m/z 667 corresponding to $[M+H]^+$ ion. The MS^2 product ion spectra of $[M+H]^+$ provided the fragment ions $[Y_1]^+$ and $[Y_0]^+$ at m/z 521 and 273, corresponding to the loss of a rhamnose and a rhamnosyl-malonylhexose residues from the protonated molecular ion, respectively. The ion at m/z 249 due to malonylhexose cation was also observed, which is useful for the characterization of the type of acyl group. The exact location of the acyl group on the glycosidic moiety is difficult to determine just on the basis of mass spectra; however, the predominant site of bonding of the acyl group is usually the 6-position of hexose, although other positions should not be excluded [73,74]. In the lower mass spectra area, the fragment ions $[B_1]^+$ at m/z 147, indicating that the molecule contains a terminal rhamnose unit, and $[B_2]^+$ at m/z 395, suggesting an acylated *O*-diglycoside, were also observed. MS^2 product ion spectra of $[Y_0]^+$ (m/z 273) confirms the identity of these aglycones as naringenin by comparison with other naringenin standards.

3.1.3. Flavanone-O-triglycosides

MS^1 scan spectra and UV spectrum lead readily to the determination of compounds **10**, **11**, **14** and **24** (RT 47.1, 47.6, 53.2 and 64.0 min) as flavanone triglycosides, explaining their early elution related to diglycosides. On the basis of m/z values, these flavonoids should be dihexosyl-rhamnosides of naringenin (**10** and **14**), eriodictyol (**11**) and hesperetin (**24**) (Table 3 in ESM). UV and MS^2 product ion spectra of $[Y_0]^+$ confirmed the identity of these aglycones by comparison with other naringenin, eriodictyol and hesperetin standards. The most probable glycosylation positions for naringenin are 4′ and 7; for eriodictyol, 3′, 4′ and 7; and for hesperetin, 3′ and 7. The presence of $[B_2]^+$ in the MS^2 product ion spectra of $[M+H]^+$ for compounds **10**, **11** and **14** suggests a diglycoside unit rhamnosyl–hexose. Since the hexose is easily lost in both positive and negative modes, these compounds are probably *O*-glycosylated with a hexose at *C*-4′ or C-3′ for hesperetin and a rhamnosyl-hexose residue (typical in *Citrus*) at *C*-7 (Fig. 1B). Hexoses are probably glucoses because it is by far the most common hexose in *Citrus* flavonoids.

The compounds **10**, detected in sweet orange, tangerine and grapefruit juices, and **14**, present in grapefruit juices, are isomers. Their MS^2 product ion spectra of $[M+H]^+$ were almost identical. The intensity of $[Y^{4'}{}_0Y^7_0]^+$ for flavanone **10** is somewhat higher than that of $[Y^{4'}{}_0Y^{7*}]^+$, however, for flavanone **14** is clearly more intense, so the first one should be the rutinoside whereas the second one, the neohesperidoside (Fig. 3) [72]. All these facts suggest that the first flavonoid is naringenin-7-*O*-rutinoside-4′-*O*-glucoside and the second one naringenin-7-*O*-neohesperidoside-4′-*O*-glucoside. These identities are consistent with the elution order (rutinosides before neohesperidosides) [19] and literature data [31,33–35,42,43]. In the same way, according to the fragmentation pattern of *O*-triglycoside flavonoids, the compound **11,** present in lemon juices, was also tentatively identified as eriodictyol-7-*O*-rutinoside-4′-*O*-glucoside and the compound **24,** detected in sweet orange and tangerine juices, as hesperetin-7-*O*-rutinoside-3′-*O*-glucoside ($[Y^{3'}{}_0Y^{7*}]^+ > [Y^{3'}{}_0Y^7_0]^+$). Other authors already reported compounds eriodictyol-7-*O*-rutinoside-4′-*O*-glucoside in leaves of *Citrus* [28] and hesperetin-7-*O*-rutinoside-3′-*O*-glucoside in orange juices [48].

3.2. Flavones

3.2.1. Flavone-O-diglycosides

Five compounds were detected within this group (Table 4 in ESM). Three of them, were identified as apigenin-7-*O*-rutinoside (compound **47**, RT 93.0 min), apigenin-7-*O*-neohesperidoside (compound **51**, RT 98.6 min) and diosmetin-7-*O*-rutinoside (compound **52**, RT 99.6 min) by comparison with standards. Apigenin-7-*O*-rutinoside, detected in tangerine, lemon and grapefruit juices, was found previously in tangerine (*Citrus unshiu* Marc), pummelo (*Citrus grandis*) [65] and leaves of sour orange (*Citrus aurantium*) and sweet orange [42]. Apigenin-7-neohesperidoside was detected in grapefruit juices according to bibliographic sources [29,35] and diosmetin-7-*O*-rutinoside, detected in tangerine in lemon juices, was found in pummelo juices [29], sweet orange and sweet orange peel [36], lemon juices [5,67], *C. lemon* × *C. sinensis* hybrid juices [41] by other authors.

Compounds **37** (RT 80.3 min) and **49** (RT 95.5 min), found in tangerine and lemon juices, were identified as 7-*O*-rutinosides of luteolin [56,57] and chrysoeriol [66], respectively, according to literature. MS^2 product ion spectra of $[M+H]^+$ of these compounds at collision energy of 10 eV showed the $[M+H]^+$ ion and the product ions $[Y_1]^+$ and $[Y_0]^+$, which correspond to the losses of rhamnose and rhamnosylglucose residues, respectively. The relative intensities of $[Y_1]^+$ and $[Y_0]^+$ ions allowed to differentiate between flavone rutinosides and neohesperidosides [72]: if $[Y_1]^+ \gg [Y_0]^+$, the flavone should be an 1→6 diglycoside (rutinoside) at C7; and, when $[Y_0]^+ \gg [Y_1]^+$, the flavone should be an 1→2 diglycoside (neohesperidoside) at C7. The aglycone identity was determined using MS^2 product ion spectra of $[Y_0]^+$ and fragmentation patterns of these aglycones, previously reported [19] (Table 4 in ESM, Fig. 2). The aglycone of compound **49** was assigned as chrysoeriol because of their earlier elution vs diosmetin-7-rutinoside and due to the lower intensity of the fragment $[Y_0–CH_3]^{+\bullet}$ in the MS^2 spectra of $[Y_0]^+$ in relation to that corresponding to its isomer diosmetin. This difference is caused by the higher stabilization of the resulting radical when the methoxy substituent is in 4′ position (diosmetin) instead of in 3′ position (chrysoeriol) [30]. Behavior which has been confirmed with the chrysoeriol and diosmetin standards.

3.2.2. Flavone-O-triglycosides

MS^1 scan spectra and UV spectra lead readily to the identification of compounds **18** (RT 56.3 min) and **21** (RT 58.2 min) as apigenin and luteolin triglycosides, respectively. MS^1 scan spectra in positive and negative ionization modes show intense protonated and deprotonated molecular ions at *m*/*z* 741 (ESI+) and 739 (ESI−) for the apigenin triglycoside and 757 (ESI+) and 755 (ESI−) for the luteolin triglycoside. This fact pointed out to dihexosyl-rhamnosyl triglycosides. MS^2 product ion spectra of $[Y_0]^+$ confirmed the identity of the aglycones by comparison with apigenin and luteolin standards (Table 5 in ESM). ESI(+)-MS/MS product ion spectra of $[M+H]^+$ elucidated the glycosylation pattern. The most usual glycosylation positions for apigenin and luteolin are 4′ and 7. Since the hexose is easily lost in both positive and negative modes, flavonoid **21** is probably *O*-glycosylated at C-4′ with a hexose and at C-7 with a rhamnosyl-hexose residue (typical in *Citrus*), just like for flavanone triglycosides (compounds **10**, **11** and **14**). Relative intensities of $[Y_1^7]^+$ and $[Y^{4'}{}_0Y_0^7]^+$ ions (Table 5 in ESM) showed that fragmentation Y_1^7 is favored against Y_0^7 in the case of flavonoid **18** and the opposite occurs for compound **21**, pointing out that the first one is a rutinoside and the last one a neohesperidoside as it has been observed for flavone diglycosides (Table 4 in ESM). According to this, compound **18,** detected in lemon juices, was tentatively identified as apigenin-7-*O*-rutinoside-4′-*O*-glucoside and compound **21,** detected in grapefruit juices, as luteolin-7-*O*-neohesperidoside-4′-*O*-glucoside, respectively. Both of them were identified in *Citrus* juices for the first time in this work.

3.2.3. Flavone mono-C-glycosides

In *C*-glycosides, the major fragmentation pathways concern cross-ring cleavages of the saccharide residue and the loss of molecules of water [75–77] (Fig. 1C). To date, C-linked sugars have only been found at the C-6 and/or C-8-positions of the flavonoid nucleus [78].

Thus, compounds **35** and **42** (RT 79.2 and 85.5 min), present in extracts of lemon and tangerine juices, were identified as diosmetin-8-*C*-glucoside and diosmetin-6-*C*-glucoside. The UV–visible spectra of these compounds showed spectra typical of flavones and the MS^1 spectra revealed high intensity $[M+H]^+$ and $[M-H]^-$ ions at *m*/*z* 463 and 461, *C*-glycoside characteristic losses of water molecules and $[^{0,2}X]^+$ and $[^{0,1}X]^+$ ions appearing at −120 and −150 *u* from $[M+H]^+$, indicating that they are *C*-glucosides (Table 6 in ESM). The ESI(+)–MS/MS spectra of $[M+H]^+$ corresponding to flavonoid *C*-glycosides usually need higher collision energies than those of *O*-glycosides since they do not possess any labile bond, and the main fragmentation pathways take place in the sugar, which has the weakest simple bonds in the molecule. Differentiation between 6*C*- and 8*C*-glucoside isomers was accomplished using the ratio of $[^{0,1}X]^+$ and $[^{0,2}X]^+$ ion intensities in the spectra at 40 eV [77]: a $[^{0,1}X]^+/[^{0,2}X]^+$ ratio near to 1:1 for 8-*C*-isomers (compound **35**) and 2:1 for 6-*C*-isomers (compound **42**). Furthermore, the presence of $[M+H-4H_2O]^+$ ion is characteristic of 6*C* isomers, whereas that of $[^{0,3}X]^+$ ion is diagnostic for 8*C* isomers [77] (Table 6 in ESM). According to literature, diosmetin-6-*C*-glucoside was previously detected in peel of Navel Orange [36], lemon juice [40] lemon peel [44]; and diosmetin-8-*C*-glucoside in leaves of sweet orange [42] and bergamot (*Citrus bergamia*) [55].

The same fragmentation pattern was observed for compounds **23** (RT 63.8 min, detected in lemon juices) and **27** (RT 68.5 min, detected in sweet orange and tangerine juices), which were identified as luteolin-6-*C*-glucoside and apigenin-8-*C*-glucoside, respectively, by comparison with reference standards. Luteolin-6-*C*-glucoside was identified in *Citrus* for the first time in this work whereas apigenin-8-*C*-glucoside was reported in the previous literature in leaves of sweet orange [42], bergamot [49] and flavedo of pummelo [29].

3.2.4. Flavone di-C-glycosides

The same mass spectral behavior of mono-*C*-glycosides was also observed for other chromatographic peaks (compounds **9**, **12**, **17** and **20**) with earlier elution and higher molecular mass, suggesting di-*C*-glycosides. Their MS^1 spectra in negative mode exhibit a high intensity $[M-H]^-$ ion, whereas, in the positive mode, the $[M+H]^+$ ion stands out. The MS^2 product ion spectra of $[M+H]^+$ were complex and showed many peaks (Table 7 in ESM). As structures are the same as in mono-*C*-glycosides (Fig. 1D), the same cleavages and losses are observed; loss of one, two, three and four molecules of water, $[^{0,2}X]^+$, $[^{0,1}X]^+$, $[^{0,4}X-2H_2O]^+$, $[^{0,3}X]^+$ cleavages and losses of one molecule of formaldehyde and two or three molecules of water, but, as there are two sugar residues, two simultaneous cleavages can occur in both sugars. In this way, many combinations are possible so the number of fragments is higher. Both diagnostic fragments $[M+H-4H_2O]^+$ for *C*-6 and $[^{0,3}X]^+$ for *C*-8, are present. These compounds were characterized as 6,8-di-*C*-glucosides of apigenin (compound **12**), chrysoeriol (compound **17**, eluting earlier than its isomer compound **20**) [30], diosmetin (compound **20**) and luteolin (compound **9**), respectively. The base

peak in the spectrum at 20 eV was the $[^{0,2}X–H_2O]^+$ ion. Since it has been observed very weak (relative abundance < 5) for mono-*C*-glycosides, this ion serves as a good diagnostic ion for di-*C*-glycosides. Most ions of the spectrum at 40 eV are caused by two simultaneous cleavages occurring in both sugars. According to literature, apigenin-6,8-di-*C*-glucoside were previously detected in sweet orange peel [36], sweet orange, tangerine [37], lemon [5,27,38,39], grapefruit juices [40] and orange–lemon hybrids juices [41]; diosmetin-6,8-di-*C*-glucoside in sweet orange peel [36], tangerine and lemon juices [5,27,38–40], lemon peel [44], pummelo (*C. grandis*) flavedo [29] and orange–lemon hybrids juices [44]; chrysoeriol-6,8-di-*C*-glucoside in lemon juices [27]; and, luteolin-6,8-di-*C*-glucoside in lemon juices [5]. As far as we know, luteolin-6,8-di-*C*-glucoside was identified in sweet orange for the first time in this work although other authors reported this compound in pummelo flavedo [29], bergamot juices [30], sour orange [31] and *Citrus myrtifolia* Raf. [32].

3.2.5. Flavone di-C-acylglycosides

In addition to flavone-6,8-di-*C*-glycosides, acylations of these compounds were detected for the first time in lemon juices at RT 71.5 and 72.8 min compounds **28** and **30**, respectively, which are isomers (Table 7 in ESM). The MS^1 spectra revealed high intensity $[M+H]^+$ and $[M-H]^-$ at *m*/*z* 799 and 797, whereas the MS^2 product ion spectra of $[M+H]^+$ show the same fragmentation pattern of chrysoeriol and diosmetin 6,8-di-*C*-glucosides: loss of water molecules from the protonated molecular ion and fragment ions $[^{0,2}X]^+$ and $[^{0,2}X-H_2O]^+$ at *m*/*z* 505 and 487. Because the protonated molecular ions are observed at *m*/*z* 799, and fragment ions with the same *m*/*z* than the chrysoeriol and diosmetin 6,8-di-*C*-glucoside fragments are detected, coming from the intramolecular cleavage of the acylated glucose or from the two sugars simultaneously, it can be concluded that these compounds contained two hexoses attached in position 6-*C* and 8-*C* of chrysoeriol and diosmetin, being one of them acylated. The joint position of the acyl group could be the position 6 of the hexose residue. This acylation was also confirmed by the presence of fragments corresponding to the only breakage of the non-acylated glucose $[^{0,2}X^{non\text{-}a}]^+$, $[^{0,2}X^{non\text{-}a}–H_2O]^+$, $[^{0,2}X^{non\text{-}a}–2H_2O]^+$, $[^{0,1}X^{non\text{-}a}]^+$, $[^{0,1}X^{non\text{-}a}–H_2O]^+$ and $[^{0,1}X^{non\text{-}a}–2H_2O]^+$.

3.2.6. Flavone O,C-glycosides

Whereas the protonated *O*-glycosides gave rise to $[Y_1]^+$ and $[Y_0]^+$ ions and the *C*-glycosides provided $[M+H]^+$ ions together with cross-ring cleavages and the loss of water molecules of the saccharide residue, the *O,C*-diglycosides presented both type of fragmentations (Fig. 1) [18,19].

In this way, two apigenin-*C*-hexosyl-*O*-pentosides (compounds **31** and **33**, RT 73.6 and 75.1 min), present in sweet orange, lemon and grapefruit juices, and one apigenin-*C*-hexosyl-*O*-hexoside (peak **32** RT 74.7 min), present in grapefruit juices, were identified (Table 8 in ESM). The ESI(+)-MS/MS spectrum from the protonated flavones **31** and **33** at low collision energy (20 eV) showed a very intense peak at *m*/*z* 433, due to the cleavage of *O*-glycosidic linkage $[Y_1]^+$, and weak fragment ions, due to the subsequent loss of water molecules and intramolecular fragmentation of *C*-glycosidic sugar from $[Y_1]^+$ ion. At high collision energy (40 eV), they presented a striking similarity to the equivalent spectrum of the corresponding mono-*C*-glycoside, showing the intense fragment ions from $[Y_1]^+$ ion [19]. Also the spectrum at 20 eV for compound **33** shows a more intense $[Y_1]^+$ ion than that for compound **31**, which indicates that the compound **33** could be the 6*C*-isomer, more difficult to fragment than the 8*C*, compound **31**. At 40 eV showed the diagnostic $[^{0,3}X]^+$ ion for 8*C*-glycosides in the compound **31**, and the diagnostic $[M+H–4H_2O]^+$ ion for 6*C*-glycosides in the compound **33** as well as a $[^{0,1}X]^+/[^{0,2}X]^+$ ratio higher. These facts are also consistent with the elution order, eluting the 8*C* before than the 6*C*-isomer, allowing to identify the compounds **31** and **33** as apigenin-8-*C*-glucoside-*O*-pentoside and apigenin-6-*C*-glucoside-*O*-pentoside, respectively. These fragmentation rules were also applied to compound **32**, found in grapefruit juices, which was characterized as apigenin-6-*C*-hexoside-*O*-hexoside. Apigenin-8-*C*-glucoside-2″-*O*-xyloside was previously detected in leaves of sweet orange and sour orange [42,79], sweet orange peel [80,81] and apigenin-8-*C*-glucoside-2″-*O*-glucoside in Hawthrorn leaf [82].

3.2.7. Flavone O,C-acylglycoside

One acylated *O,C*-glycosylated apigenin was also detected at RT 91.6 min (compound **45**) in sweet orange and grapefruit juices for the first time in this work. This compound showed a characteristic UV–visible spectrum of apigenin flavonoid. The MS^1 spectra in both ionization modes gave rise to an intense $[M+H]^+$ ion (*m*/*z* 709, base peak). The MS^2 product ion spectra of $[M+H]^+$ showed identical fragmentation profile as apigenin-8-*C*-glucoside-*O*-pentoside, except for the spectrum at lower collision energy, in which $[Y_1]^+$ ion was observed at *m*/*z* 433 due to the loss of an acylglycoside residue (-276 u) of unknown identity. In addition, the fragment ion corresponding to the acylglycoside residue was observed at *m*/*z* 277 (Table 8 in ESM).

3.3. Flavonols

3.3.1. Flavonol-O-diglycosides

Three flavonol diglycosides were characterized as quercetin-3-*O*-rutinoside (compound **41**) and isorhamnetin-3-*O*-rutinoside (compound **54**) at retention time 83.2 and 104.8 min in sweet orange, tangerine and lemon juices; and as kaempferol-3-*O*-rutinoside (compound **53**) at 99.8 min in sweet orange, tangerine and grapefruit juices by comparison with reference standards. MS^2 product ions spectra of $[M+H]^+$ ion for all three compounds showed identical fragmentation pattern: $[Y_0]^+$ ion is similar to or slightly more abundant than the $[Y_1]^+$ ion and a weak $[Y^*]^+$ ion (Table 9 in ESM), being the first fact diagnostic of flavonol-3-*O*-rutinosides [72]. Other authors already reported quercetin-3-*O*-rutinoside in lemon juices [40,62], grapefruit juices [29,58], leaves and peels of sweet orange [63] and isorhamnetin-3-*O*-rutinoside in Citrumelo CPB 4475 (*C. paradise* L. Macf. X *Poncirus trifoliata* L. Raf.) and Carrizo Cintrage (*C. sinensis* L. Osb. × *P. trifoliata* L. Raf.) [28].

In addition, a quercetin diglycoside characterized as quercetin-7-*O*-rutinoside (compound **29**) at 72.2 min in juices of the four studied *Citrus* fruits, a kaempferol diglycoside designated as kaempferol-7-*O*-rutinoside (compound **44**) at 88.7 min in tangerine juices, and a isorhamnetin and a tamarixetin diglycosides identified tentatively as 7-*O*-rutinoside of isorhamentin and tamarixetin (compounds **48** and **50**) at 93.0 min in sweet orange, tangerine and lemon juices and at 96.2 min in tangerine juices, respectively, were also found for the first time in this work (Table 9 in ESM). The isorhametin and tamarixetin aglycones are isomers and presented the same MS^2 product ion spectra. The identity of these aglycones was elucidated tentatively according to elution order, isorhamnetin before tamarixetin [83,84]. In contrast to the detected flavonol-3-*O*-rutinosides, in the MS^2 product ion spectra of $[M+H]^+$ for these four flavonols, the $[Y_1]^+$ fragment was considerably more intense than $[Y_0]^+$ ion, characteristic of 7-*O*-rutinosides [72]. In addition, despite being observed $[Y^*]^+$, this is very low intensity in the same way than other studied flavone-7-*O*-rutinosides. The aglycone identity was confirmed by comparison of the MS^2 products ion spectra of $[Y_0]^+$ with their respective standards (Fig. 2).

3.3.2. Flavonol-O-triglycosides

Single stage MS experiments and UV–visible spectra lead readily to the determination of the compounds **7**, **13**, **16** and **19** (RT 33.7, 49.4, 54.3 and 56.8 min, respectively) as flavonol triglycosides, which explains their early elution. On the basis of *m*/*z* values, these flavonoids should be dihexosyl-rhamnosyl-flavonols (Table 1).

Glycosylation pattern was tentatively determined by MS^2 product ion spectra of $[M+H]^+$. Major fragments are $[M+H-rha]^+$, loss of a rhamnose residue due to the cleavage of the interglycosidic bond rhamnose–hexose from the disaccharide in 3 position ($[Y_1^3]^+$); $[M+H-rha-hex]^+$, due to the loss of the complete diglycoside at C-3 ($[Y_0^3]^+$) or to the cleavage of the hexose–aglycone bond in 7 position and of the rhamnose at C-3 ($[Y_1^3Y_0^7]^+$); and, $[M+H-rha-2hex]^+$, the loss of glycans at both positions ($[Y_0^3Y_0^7]^+$) (Table 10 in ESM). A very weak ion $[Y^{3*}]^+$, which can be due to the loss of the internal dehydrated hexose residue of the diglycoside from the $[M+H]^+$ ion, just as in flavonoid rutinosides; and the ion $[Y^{3*}Y_0^7]^+$ due to the simultaneous loss of two hexoses, the internal dehydrated hexose residue from the diglycoside and the hexose linked to 7 position of the aglycone, were also observed. The diglycoside seems to be bounded in position 3 of the aglycone because the intensities of $[Y_1^3]^+$ and $[Y_0^3]^+$ ions are similar [72]. All these facts suggest that these flavonoids are 3-*O*-rutinoside-7-*O*-glucosides.

MS^2 product ion spectra of $[Y_0]^+$ ion confirm the identity of the aglycones as quercetin (compound **7**), kaempferol (**13**), isorhamnetin (**16)** and tamarixetin (**19**) by comparison with standards. Thus, the compound **7,** present in sweet orange, tangerine and lemon juice, was identified as quercetin-3-*O*-rutinoside-7-*O*-glucoside, which was previously detected in lemon juice and tree [27], leaves of other *Citrus* [28], and the compound **13,** present in orange and tangerine juices, as kaempferol-3-*O*-rutinoside-7-*O*-glucoside, which was reported in leaves of other *Citrus* [28]. As far as we know, the compound **16,** detected in sweet orange, tangerine and lemon juices, and the compound **19**, present in tangerine juices, were characterized as isorhamnetin-3-*O*-rutinoside-7-*O*-glucoside and tamarixetin-3-*O*-rutinoside-7-*O*-glucoside, respectively, in *Citrus* for the first time in this work.

Compound **15** (RT 53.6 min), present in sweet orange and tangerine juices, characterized as isorhamnetin-3-*O*-hexoside-7-*O*-rhamnosylhexoside for the first time in *Citrus*, showed just the opposite MS fragmentation pattern. MS^2 product ion spectra of $[M+H]^+$ ion exhibited a very intense $[Y_0^3]^+$ ion, formed by the glycosidic bond cleavage and elimination of the hexose residue. Since the favoured fragmentation position is the C3 hydroxyl, it was suggested that the monosaccharide was linked to this group and the diglycoside to the 7 position.

This same behavior was observed in other two flavonol triglycosides, which were identified as quercetin-3-*O*-rhamnoside-7-*O*-rhamnosylhexoside (compound **26**, RT 67.7 min) and isorhamnetin-3-*O*-rhamnoside-7-*O*-rhamnoylhexoside (compound **38**, RT 80.0 min) in sweet orange and tangerine juices for the first time. MS^2 product ion spectra of $[M+H]^+$ of these compounds showed an intense $[M+H-rha]^+$ ion, explained by two competitive fragmentation paths, the one involving the cleavage of the monosaccharide–aglycone glycosidic bond ($[Y_0^3]^+$) and the other the cleavage of the interglycosidic bond rhamnose–hexose ($[Y_1^7]^+$). Due to the absence of $[Y^{3*}]^+$ ion, typical in rhamnosylhexosides, the rhamnose monosaccharide should be attached to 3 position and the disaccharide to 7 position of the aglycone. This fact is supported by the detection of $[Y_0^3Y_1^7]^+$ ion due to the rupture of the rhamnose–aglycone glycosidic bond in 3 position and the rhamnose–hexose interglycosidic union in 7 position, and a weak $[Y_0^3Y^{7*}]^+$ ion corresponding to the cleavage of the glycosidic bond in 3 position and the loss of the internal dehydrated hexose residue from the disaccharide in 7 position.

On the other hand, compound **36** (RT 79.1 min) was characterized as kaempferol-3-*O*-rhamnosylhexoside-7-*O*-rhamnoside in sweet orange and tangerine juices for the first time. MS^2 product ion spectra of $[M+H]^+$ ion showed the $[Y^{3*}]^+$ ion, corresponding to the loss of the internal dehydrated hexose residue of diglycoside from $[M+H]^+$, indicating that the disaccharide hexose–rhamnose residue is attached to 3 position and the monosaccharide to 7 position of the aglycone.

3.4. Dihydroflavonol-O-diglycosides

Three flavonoids (compounds **2**, **5** and **8**) with flavanone like UV–vis spectra were detected at retention times of 21.2, 27.1 and 36.2 min in studied *Citrus* juices (Table 1). MS^1 spectra show the $[M+H]^+$ and $[M-H]^-$ ions at *m*/*z* 613, 597 and 627, and 611, 595 and 625, respectively, together with their sodium and bisulfate adducts. Their earlier retention times versus flavanones and the two mass units differences in MS spectra related to their flavonol homologues suggest their identification as dihydroflavonols. MS^2 product ion spectra of $[M+H]^+$ were very similar to those of flavanone-7-*O*-rutinosides and revealed a 1→6 interglycosidic linkage (rutinosides) [72] ($[Y_1]^+$ not » $[Y_0]^+$, $[Y^*]^+$ intense and $[Y^*]^+ \geq [Y_0]^+$) (Table 11 in ESM). Finally, MS product ion spectra of the protonated aglycones ($[Y_0]^+$) allowed their identification as dihydroquercetin-7-*O*-rutinoside (compound **2**), dihydrokaempferol-7-*O*-rutinoside (compound **5**) and dihydroisorhamnetin-7-*O*-rutinoside (compound **8**) (Table 12 in ESM) (Fig. 2) [26].

3.5. Hydroxycinnamic acids

Two chromatographic peaks (compounds **4** and **6**, RT 25.2 and 27.3 min) with the same UV spectra as the standards of ferulic and sinapic acids were detected for the first time in *Citrus* juices (Fig. 2). They showed the $[M-H]^+$ and $[M-H]^-$ ions at *m*/*z* 357 and 355 and 387 and 385, respectively. ESI(+)-MS/MS product ion spectra of $[M+H]^+$ for these acids yielded only the fragment ions [ferulic acid+H]$^+$ and [ferulic acid+H−H_2O]$^+$ at *m*/*z* 195 and 177 for compound **4** and [sinapic acid+H]$^+$ and [Sinapic acid+H−H_2O]$^+$ at *m*/*z* 225 and 207 for compound **6**, corresponding to the loss of one hexose from the $[M+H]^+$ ion and the successive loss of one water molecule (Table 13 in ESM). These ions indicate that both compounds are probably *O*-hexosides of ferulic acid and sinapic acid. At high CE other secondary ions appeared, that are characteristic of the fragmentation patterns of these acids in their free form [19].

3.6. Coumarins

Two scopoletin derivates (compounds **1** and **3**, RT 20.5 and 24.9 min) were detected in grapefruit juices for the first time (Fig. 2). These compounds showed the same UV spectrum as scopoletin standard and the protonated molecular ions at *m*/*z* 355 (peak 1) and 501 (peak 3) in positive MS^1 spectra. The ESI(+)-MS/MS spectra of $[M+H]^+$ of these coumarins revealed the presence of $[Y_n]^+$ product ions in addition to the $[M+H]^+$ ion (Table 14 in ESM). Thus, the compound **1** was tentatively characterized as scopoletin-*O*-hexoside due to the intense $[Y_0]^+$ ion at *m*/*z* 193, corresponding to the protonated scopoletin, and the $[B_1]^+$ ion at *m*/*z* 163 from the hexoside. In the same way, the compound **3** was identified as scopoletin-*O*-rhamnosylhexoside, showing the $[Y_1]^+$ and $[Y_0]^+$ ions at *m*/*z* 359 and 193, respectively, in addition to the $[B_1]^+$ and $[B_2]^+$ ions at *m*/*z* 147 and 308 that indicate a rhamnosylhexoside structure. The ESI(+)–MS/MS product ion spectra of $[Y_0]^+$ confirmed the identity of the aglycone by comparison with the scopoletin standard [19].

4. Conclusions

The most important contribution of this work is the comprehensive characterization of phenolic compounds in *Citrus* juices (sweet orange, tangerine, lemon and grapefruit) from Spanish cultivars by HPLC-DAD-ESI-CID-MS/MS. The structural information provided by online technical HPLC-DAD-ESI-CID-MS/MS scan and product ion scan mode led to identify and characterize successfully 58 phenolic compounds in *Citrus* fruit juices using the mechanisms and fragmentation patterns established in the previous study with phenolic compounds standards. Although some of the phenolic compunds have been previously described in literature, others have been detected for the first time in *Citrus* in this work. Flavanone: *O*-dihexoside of naringenin; flavones: apigenin-7-*O*-rutinoside-4′-*O*-glucoside, luteolin-7-*O*-neohesperidoside-4′-*O*-glucoside, luteolin-6-*C*-glucoside, 6,8-di-*C*-acylhexosides of chrysoeriol and diosmetin, 6*C*- and 8*C*-glucoside-*O*-pentoside of apigenin, apigenin-6-*C*-hexoside-*O*-hexoside and apigenin-8-*C*-hexoside-*O*-acylrhamnoside; flavonols: 7-*O*-rutinosides of quercetin, kaempferol, isorhamnetin and tamarixetin, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside-7-*O*-glucoside, tamarixetin-3-*O*rutinoside-7-*O*-glucoside, isorhamnetin-3-*O*-hexoside-7-*O*-rhamnosylhexoside, 3-*O*-rhamnoside-7-*O*-rhamnosylhexoside of quercetin and isorhamnetin and kaempferol-3-*O*-rhamnosylhexoside-7-*O*-rhamnoside; hydroxycinnamic acids: *O*-hexoside of ferulic and sinapic acid; and coumarins: *O*-hexoside and *O*-rhamnosylhexoside of scopoletin.

Thus, this paper shows how the proposed rationalized methodology for identification of phenolic compound described in a previous work is applied for the successful characterization of the whole polyphenolic profile found in *Citrus* fruit juices from Spain. The more phenolic compound MS spectra are studied and reported, the more accurate and applicable rules and guidelines can be developed. In this way, this study exemplifies how these guidelines can be used for phenolics different from the original standards.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.05.042.

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