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On-line characterisation of apple polyphenols by liquid chromatography coupled with mass spectrometry and ultraviolet absorbance detection

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Abstract

Apple polyphenols were characterised by means of hyphenated techniques such as HPLC coupled to UV photodiode array detection (LC-DAD) and to mass spectrometry (LC–MS). LC–MS using atmospheric pressure ionisation (APCI) in the positive ion mode provided the molecular weight, the number of hydroxyl groups, the number of sugars and an idea about the substitution pattern of the polyphenols. LC-DAD with postcolumn addition of UV shift reagents afforded precise structural information about the position of the free hydroxyl groups in the polyphenolic nucleus. Five isorhamnetin glycosides, two hydroxyphloretin glycosides and quercetin were reported in apple peel for the first time. Postcolumn addition of UV shift reagents in LC-DAD analysis confirmed the presence of isorhamnetin glycosides and not the isomeric glycosides of rhamnetin. Moreover, isorhamnetin-3-*O*-rhamnoglucoside was identified unambiguously by comparison with a standard. These results are relevant not only from a chemotaxonomic point of view, but also in the control of authenticity of fruit derived products in order to detect fraudulent admixtures.

Keywords: Apples; Derivatization, LC; Polyphenols

1. Introduction

Polyphenols are major constituents of apples and play an important role in the nutritional, organoleptic and commercial properties of these fruits and their derived products. In addition, polyphenols are natural antioxidants present in human diet that have a great interest because of their health protective properties against cardiovascular diseases [1] and cancer [2]. Phenolic compounds are responsible for bitterness, astringency and flavour of apples and their derived foodstuffs [3]. Moreover, they are involved in the formation of hazes and sediments in apple juices and ciders [4], and in the browning phenomena contributing to their colour [5]. In cidermaking processes, polyphenols are largely implicated, providing cider aroma [6], controlling fermentation rates [7], avoiding

Abbreviations: AVI, quercetin-3-*O*-arabinofuranoside (avicularin); CA, 5-caffeoylquinic acid; CAT, (+)-catechin; FA-1, FA-2, FA-4, FA-6, FA-7, flavan-3-ol dimers; FA-3, FA-5, flavan-3-ol trimers; EC, (−)-epicatechin; HYP, quercetin-3-*O*-galactoside (hyperoside); IDE, cyanidin-3-*O*-galactoside (ideain); IQC, quercetin-3-*O*-glucoside (isoquercitrin); ISR-1, ISR-4, ISR-5, ISR-6, unknown isorhamnetin glycosides; ISR-2, isorhamnetin-3-*O*-glucoside; ISR-3, isorhamnetin-3-*O*-ramnoglucoside; PB2, procyanidin B2; PCQ, 4-*p*-coumaroylquinic acid; PLD-1, hydroxyphloretin diglycoside; PLD-2, hydroxyphloretin monoglycoside; PLG, phloretin-2′-*O*-glucoside (phloridzin); PLXG, phloretin-2′-*O*-xyloglucoside; PPO, polyphenoloxidase; QCE, quercetin; QCI, quercetin-3-*O*-ramnoside (quercitrin); QC-C5, unknown quercetin glycoside; RUT, quercetin-3-*O*-rhamnoglucoside (rutin)

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the development of some faults in cider [8], participating in cider spontaneous clarification and inhibiting enzymatic systems such as clarification enzymes [9].

Knowledge of the precise composition of apple varieties may contribute to a better understanding of their influence in the quality and diversity of apple products such as apple juice and cider. For this reason, characterisation studies based on the polyphenolic profiles have been carried out with dessert and cider apple cultivars [10–14]. The main classes of polyphenols found in apple fruit are flavan-3-ols (catechins (monomeric flavan-3-ols) and procyanidins (polymeric flavan-3-ols)), dihydrochalcones (phloretin glycosides), flavonols (quercetin and isorhamnetin glycosides), hydroxycinnamic acids and anthocyanins (cyanidin glycosides) (Fig. 1).

Polyphenols are considered to be useful chemotaxonomic makers [15], as well as an adequate tool for establishing food authenticity criteria. In this sense, these compounds are of value for the detection of adulteration of fruit juices [16], jellies [17], jams [18] and purees [19]. Among apple polyphenols, phloretin derivatives have been generally thought to be specific constituents of this fruit [12], but a recent study has revealed that phloridzin is also present in strawberry fruits [20]. In pear fruit, isorhamnetin glycosides have been suggested as suitable markers [16,21]. However, isorhamnetin-3-*O*-glucoside and another hexose of isorhamnetin have been detected in apple peel and in similar concentrations to those found in pear [22]. These observations are of remarkable importance with regard to the detection of fraudulent admixtures. Thus, neither isorhamnetin glycosides by themselves nor phloridzin are sufficient for the detection of apple juice adulterated with pear juice, as was previously proposed. In this paper, the presence of five isorhamnetin glycosides, two hydroxyphloretin derivatives and quercetin aglycone in apple peel extract are reported for the first time. In addition, other known apple polyphenolic constituents are confirmed.

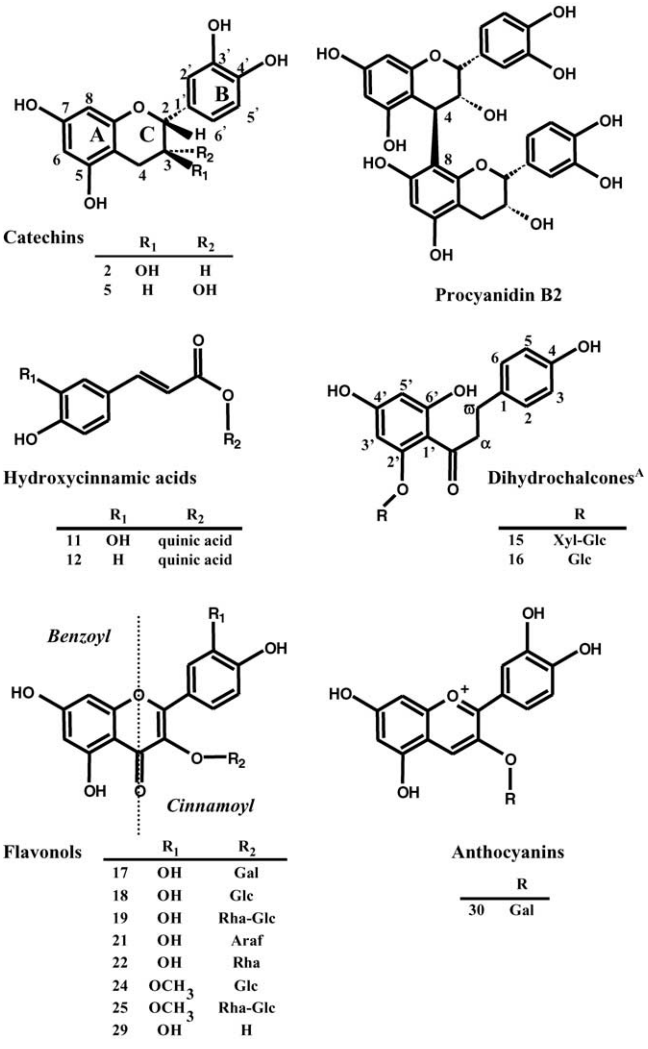

Fig. 1. Polyphenols in apple. Abbreviations: Araf, arabinofuranose; Glc, glucose; Gal, galactose; Rha, rhamnose; Xyl, xylose. 'A' denotes traditional numbering of carbons in dihydrochalcones.

High pressure liquid chromatography coupled with a photodiode-array detector (HPLC-DAD) provides extensive information on polyphenol structures in crude or semi-purified plant extracts [10–14,23]. However, the identity of non-phenolic moieties in molecules, such as sugars or aliphatic acyl groups, are not revealed by LC-DAD, because these groups do not have strong UV chromophores. Liquid chromatography coupled with mass spectrometry (LC–MS) can furnish this information on-line without previous isolation or hydrolysis of the compounds [24]. In order to achieve the on-line identification of apple polyphenols, HPLC-DAD/MS and HPLC-DAD with post-column derivatization with reagents that induce band shifts in the UV spectra of analytes have been used. HPLC–MS provides information about the polyphenol molecular weight and the molecular structure from its fragmentation data [25,26]. Ionisation was achieved through an atmospheric pressure chemical ionisation (APCI) interface. APCI is a soft ionisation technique which generates mainly protonated molecular ions in the positive mode and deprotonated molecular ions in the negative mode. It is widely applied and specially suitable for relatively small molecules with molecular weights up to 2000 Da and medium polarity, such as polyphenols [27].

HPLC-DAD with post-column addition of UV shift reagents provides rapid information about the polyphenol aglycone and its substitution pattern [28]. Shift reagents induce a displacement of the absorption maxima, which can be used to determine the position of free hydroxyl groups. The application of these shift reagents to structure elucidation of flavonoids has been extensively described [29] and successfully applied to different polyphenol families [15,30].

2. Experimental

2.1. Solvents and phenolic standards

Methanol (Romil Chemical Ltd., Heidelberg, Germany) was of HPLC grade, water was purified on a Reinstwasser

system (St-Gallen, Switzerland), and glacial acetic acid from Fluka (Buchs, Switzerland) was of analytical quality. Sodium acetate and trifluoroacetic acid (TFA) of analytical quality were provided by Merck (Darmstadt, Germany) and aluminium chloride and sodium hydroxide of analytical quality by Fluka (Buchs, Switzerland). Every solvent was filtered through a Millipore membrane (aqueous solutions: 0.45 μm HA; organic solutions: 0.50 μm FH) (Bedford, MA, USA).

Polyphenol standards were supplied as follows: (+)-catechin, (−)-epicatechin, quercetin-3-*O*-rhamnoglucoside (rutin), quercetin, phloretin-2′-*O*-glucoside and 5-caffeoylquinic acid by Sigma–Aldrich Chemie (Steinheim, Germany); quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-arabinofuranoside (avicularin), quercetin-3-*O*-ramnoside (quercitrin), quercetin-3-*O*-xyloside (reynoutrin), isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside (isorhamnetin-3-*O*-rhamnoglucoside) and cyanidin-3-*O*-galactoside (ideain) chloride by Extrasynthèse (Genay, France). 4-*p*-Coumaroylquinic acid, phloretin-2′-*O*-xyloglucoside and procyanidin B2 were kindly provided by Dr. S. Guyot, Dr. F.A. Tomás-Barberán and Dr. C. Santos-Buelga, respectively.

2.2. Samples

Cider apples of the Goikoetxea variety were harvested at the stage of maturity during the 2000 season in the experimental orchard of the Diputación Foral de Gipuzkoa (Hondarribia, Guipúzcoa). Ten fruits were mechanically peeled. Apple peel was frozen in liquid nitrogen and freeze-dried. Then, the dried peel was crushed in closed vials to avoid hydratation, obtaining an homogenous powder that was stored at room temperature in a desiccator until analysis.

Freeze-dried apple peel powder (0.5 g) was extracted with 30 mL of methanol–water–acetic acid (30:69:1, v/v/v) in an ultrasonic bath during 10 min [31]. Afterwards, the extract was freeze-dried once methanol had been evaporated at 40 °C in a rotavapor (Rotavapor Büchi RE111, Büchi 461 Waterbath, Büchi B-171 Vacobox; Flawil, Switzerland). The solution analysed was prepared by dissolving 50 mg of the extract in 1 mL of methanol–water–acetic acid (30:69:1, v/v/v).

2.3. LC-UV analysis

Separations were performed on a Nova-Pak C18 column (300 mm × 3.9 mm i.d., 4 μm) and a Nova-Pak C18 guard column (10 mm × 3.9 mm i.d., 4 μm) from Waters (Barcelona, Spain). HPLC-DAD equipment and chromatographic conditions used in each analysis are described below (Sections 2.4 and 2.5). Flavan-3-ols and dihydrochalcones were monitored at 280 nm, hydroxycinnamic acids at 320 nm, flavonols at 370 nm and anthocyanins at 530 nm. Their UV–vis spectra (DAD) were recorded between 190 and 600 nm.

2.4. LC–MS analysis

Chromatographic analyses were performed on a Hewlett-Packard Series 1100 system (Palo Alto, CA, USA) equipped with a vacuum degasser, a binary pump, an autosampler, and a photodiode-array detector (DAD), connected to HP ChemStation software (Hewlett-Packard, Palo Alto, CA, USA). Mass spectra were obtained on a Finnigan-MAT model TSQ 700 (San Jose, CA, USA) triple quadrupole mass spectrometer. Solvents for the mobile phase were A: 0.5% acetic acid in water (v/v), and B: methanol. Elution conditions applied were as follows: 0–10 min, 5%B isocratic; 10–50 min, linear gradient 5–15%B; 50–70 min, 15%B isocratic; 70–100 min, linear gradient 15–30%B; 100–125 min, linear gradient 30–60 %B; and finally, washing and reconditioning of the column. Flow rate was 0.8 mL/min and injection volume, 50 μL. The system operated at room temperature.

LC/APCI-MS analyses were performed in the positive ion mode. The APCI parameters of the source were: capillary temperature, 150 °C; vaporiser temperature, 450 °C; corona needle current, 5 μA; sheath gas pressure (nitrogen), 23 psi. Ion monitoring mode was *full scan* in the range 100–1500 amu, that provides a complete mass spectrum for each analyte.

2.5. LC-UV analysis with post-column addition of UV shift reagents

Chromatographic analyses were performed on a Hewlett-Packard Series 1050 system (Palo Alto, CA, USA) equipped with a quaternary pump, an autosampler and a photodiode-array detector (DAD), connected to HP ChemStation software (Hewlett-Packard, Palo Alto, CA, USA). Solvents were degassed on-line with helium. Solvents used as mobile phase were A: water, and B: methanol. Elution conditions applied were as follows: 0–10 min, 5%B isocratic; 10–50 min, linear gradient 5–15%B; 50–70 min, 15%B isocratic; 70–100 min, linear gradient 15–30%B; 100–125 min, linear gradient 30–60 %B; and finally, washing and reconditioning of the column. Flow rate was 0.8 mL/min and injection volume, 50 μL. The system operated at room temperature.

The method used for post-column addition of UV shift reagents is based on a previously reported protocol [25]. The post-column device consisted of two M-6000 pumps (Waters, Bedford, MA, USA), an Upchurch (Oak Harbor, WA, USA) mixing tee, a 10 μL Visco mixer (Lee, Westbrook, CO, USA) and a reaction coil (1 m × 0.5 mm i.d., 0.20 mL). Reagents used in the post-column derivatization system were the following (Table 1): weak base, 0.5 M aqueous sodium acetate solution (basified with a 0.01 M NaOH solution to pH 8); 0.3 M aqueous aluminium chloride solution (with this reagent, the reaction coil was heated to 80 °C and the eluent was previously neutralised with a 0.02 M NaOH solution); 0.3 M aqueous aluminium chloride solution with

Table 1
Experimental conditions for postcolumn addition of UV shift reagents

Shift reagent	Pump 1	Flow 1 (mL/min)	Pump 2	Flow 2 (mL/min)	pH	Temperature (°C)
Eluent	H_2O	0.2	H_2O	0.2		RT
NaOAc	NaOH 0.01 M	0.2	NaOAc 0.5 M	0.2	8	RT
$AlCl_3$	NaOH 0.02 M	0.2	$AlCl_3$ 0.3 M	0.2	7	80
$AlCl_3$ (0.1% TFA)	H_2O (0.1% TFA)	0.2	$AlCl_3$ 0.3 M (0.1% TFA)	0.2	2.5	80

RT, room temperature.

0.1% TFA (reaction coil at 80 °C and the mobile phase contained 0.1% TFA). All reagent solutions were filtered through a 0.50 μm Millipore membrane (Bedford, MA, USA). Postcolumn addition of the bases (for neutralisation of the mobile phase) and of the UV shift reagents was achieved with two pumps. Neutralisation of the mobile phase was effected in the mixing tee, and reaction with the shifts reagents was carried out in the 10 μL Visco mixer followed by the reaction coil.

3. Results and discussion

Efficient separations of apple polyphenols found in the crude peel extract were achieved with a reversed-phase RP-18 column with gradient elution systems consisting of methanol–0.5% acetic acid in water for LC/APCI-MS analysis (Fig. 2), and methanol–water for LC-UV with postcolumn addition of UV shift reagents (Fig. 3).

3.1. LC-UV photodiode array detection

A photodiode-array detector allows recording of UV–vis spectra of each peak of the chromatogram (Figs. 2 and 3) and thus, unambiguous attribution of each chromatographic peak to a certain class of polyphenols, since each class exhibits a characteristic UV–vis spectrum [28]. In this sense, UV spectra (in mobile phase: methanol–water or methanol–acidic water) of flavonols exhibit two major absorption peaks in the region 240–400 nm, commonly referred to as Band I (300–380 nm) and Band II (240–280 nm). Band I is considered to be associated with the absorption due to the B-ring cinnamoyl system, and Band II with absorption involving the A-ring benzoyl system (Fig. 1). In flavonols with a substituted 3-hydroxyl group (methylated or glycosylated) (peaks 17–29), Band I is in the region 328–357 nm. Flavan-3-ols (peaks 1–10) and dihydrochalcones (peaks 13–16) are readily distinguished from flavonols by their UV spectra; the former typically exhibit an intense Band II (275–285 nm) and do not present Band I as a result of their lacking any conjugation between the A- and B-rings. The hydroxycinnamic acid structure contains the cinnamoyl system, which is responsible for the Band I absorption (310–325 nm) seen in the UV spectra (peaks 11–12). Anthocyanins (peak 30) in acidic media exhibit two major absorption bands in the regions 275–285 and 520–545 nm [32].

3.2. Mass spectrometry coupled with high pressure liquid chromatography

Mass spectra of the polyphenols analysed in the apple peel crude extract provided data about their molecular weight and their constitutive units. For instance, among flavan-3-ols, five dimers and two trimers of (+)-catechin and/or (−)-epicatechin were detected. With regard to dihydrochalcones and flavonols, fragmentation of the *O*-glycosidic bonds provided information about the nature of their glycosides by subtracting the fragment mass from the protonated molecular ion $[M + H]^+$ mass. APCI mass spectra of polyphenols in the positive ion mode usually show a weak protonated molecular ion and a main peak corresponding to the protonated aglycone moiety $[A + H]^+$ [27]. In Table 2, mass spectral data of the polyphenols detected in apple peel extract are summarised. From the information obtained from UV–vis and mass spectra, identification of polyphenols was possible by comparison with reference substances. For other polyphenols, certain structural elements could be established.

3.2.1. Flavan-3-ols

Under the mentioned experimental conditions used for HPLC-DAD-MS analysis, several well-resolved chromatographic peaks of flavan-3-ols were observed. In the mass spectra of catechins (flavan-3-ol monomers), (−)-epicatechin (EC) and (+)-catechin (CAT) exhibit their protonated molecular ion at *m/z* 291 amu. The other flavan-3-ols detected in the crude extract were dimers (FA-1, FA-2, FA-4, FA-6 and FA-7 and PB2) and trimers (FA-3 and FA-5) of (−)-epicatechin and/or (+)-catechin, that presented peaks of major relative abundance at *m/z* 291 amu. In their mass spectra, the protonated molecular ion (dimers, at *m/z* 579 amu; trimers, at *m/z* 867 amu) and weaker ions due to the losses of monomeric units were also detected. From this observation, it could be asserted that the flavan-3-ols detected were only constituted by units of (−)-epicatechin and/or (+)-catechin, but it was not possible to specify in each case which monomers ((−)-epicatechin and/or (+)-catechin) were the constituents, or the type of linkage between their units. Only procyanidin B2 (PB2) was identified by comparison with its standard.

3.2.2. Dihydrochalcones

Four dihydrochalcones were detected, two of which were identified as phloretin-2′-*O*-glucoside (phloridzin, PLG) and

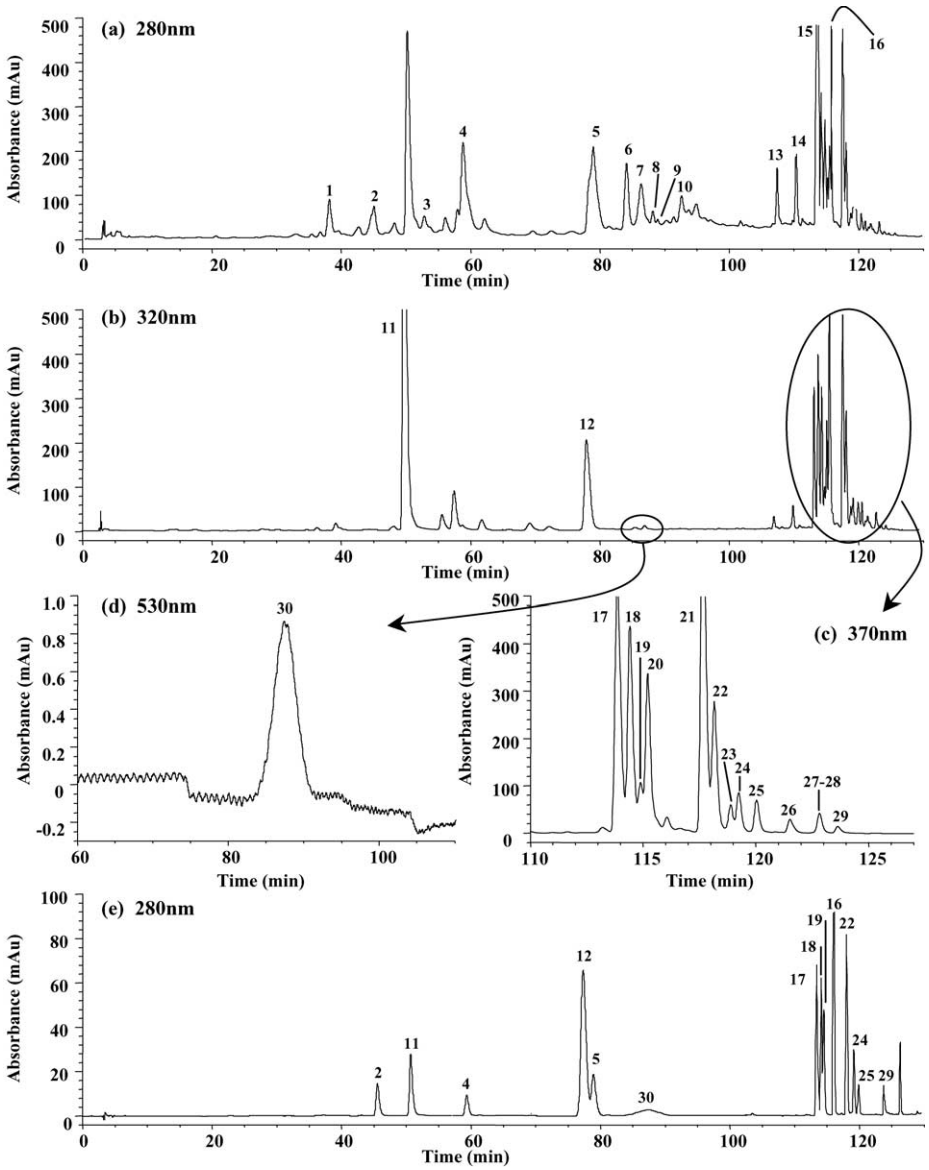

Fig. 2. Chromatographic separation of apple peel polyphenols under experimental conditions used for HPLC-DAD/APCI-MS analysis recorded at (a) 280 nm (flavan-3-ols and dihydrochalcones), (b) 320 nm (hydroxycinnamic acids), (c) 370 nm (flavonols) and (d) 530 nm (anthocyanins). (e) Chromatogram at 280 nm of a standard mixture of polyphenols. For chromatographic peak identification see Table 2.

phloretin-2′-*O*-xyloglucoside (PLXG) by comparison with standards. Their mass spectra presented protonated molecular ions at *m*/*z* 437 and 569 amu, respectively and fragments corresponding to the losses of sugar units: phloretin-2′-*O*-glucoside mass spectra showed the ion of the protonated aglycone $[A + H]^+$ at *m*/*z* 275 amu (phloretin aglycone) due to the loss of a glucosyl moiety; and phloretin-2′-*O*-xyloglucoside mass spectra presented a first loss of 132 amu corresponding to a xylosyl moiety, followed by a loss of 162 amu (glucosyl moiety) leading to the ion $[A + H]^+$ at *m*/*z* 275 amu.

Molecular weights of dihydrochalcones PLD-1 and PLD-2 were 16 amu higher than those of phloretin-2′-*O*-xyloglucoside and phloretin-2′-*O*-glucoside, respectively. This fact indicated that PLD-1 and PLD-2 contained one more hydroxyl group in their structures. Ions observed in the PLD-1 mass spectrum disclosed that it contained a diglycoside in its structure: it presented a weak intermediate fragment due to the loss of a first sugar unit (132 amu), corresponding to a pentosyl residue; and then, a loss of 162 amu (hexosyl residue) leading to the protonated aglycone ion $[A + H]^+$. In PLD-2 mass spectrum, the $[A + H]^+$ ion was generated by the loss of an hexosyl moiety. Taking into account the information provided by mass spectra, it was deduced that the additional hydroxyl group of PLD-1 and PLD-2 is located in the polyphenolic nucleus, since the mass difference of 16 amu was also found between their protonated aglycone ions.

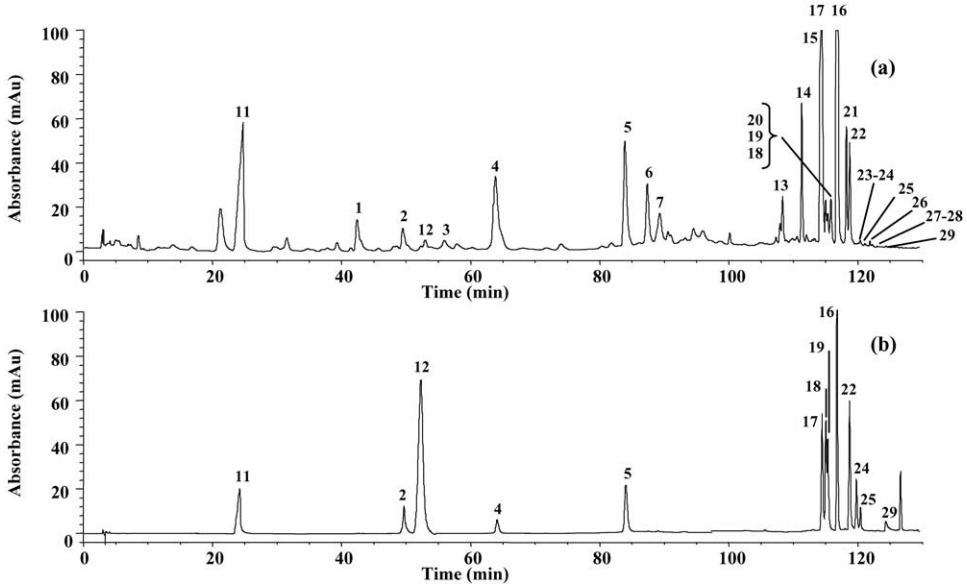

Fig. 3. Chromatographic separation of polyphenols under experimental conditions used for HPLC-DAD with postcolumn addition of UV shift reagents (280 nm): (a) apple peel extract and (b) standard mixture of polyphenols. For chromatographic peak identification see Table 2.

3.2.3. Flavonols

Among flavonols, the aglycone quercetin and some of its glycosides were detected by comparison with standards: quercetin-3-*O*-galactoside (hyperoside, HYP), quercetin-3-*O*-glucoside (isoquercitrin, IQC), quercetin-3-*O*-rhamnoglucoside (rutin or quercetin-3-*O*-rutinoside, RUT), quercetin-3-*O*-arabinofuranoside (avicularin, AVI), and quercetin-3-*O*-ramnoside (quercitrin, QCI). Moreover, another unknown quercetin glycoside (QG-C5) was also present. Quercetin, detected as free aglycone in the apple peel extract, has not been reported before, probably due to its low concentration. Quercetin monoglycoside mass spectra showed the protonated molecular ion and the protonated aglycone ion as a result of the loss of the sugar residue (losses: 132 amu, pentosyl residue; 146 amu, deoxyhexosyl residue; 162 amu, hexosyl residue). In the mass spectrum of the quercetin diglycoside (quercetin-3-*O*-rhamnoglucoside), a fragment due to the loss of the first sugar unit of 146 amu corresponding to a deoxyhexosyl was also observed. According to the mass spectrum of QG-C5, its glycosidic moiety was constituted by a pentosyl unit.

Moreover, other kind of flavonols were found in the apple peel extract: isorhamnetin glycosides. As far as is known, there is just one publication referring to the existence of this class of flavonols in apple [22]. Schieber et al. detected two isorhamnetin glycosides (hexoses) in apple peel: isorhamnetin-3-*O*-glucoside and probably, isorhamnetin-3-*O*-galactoside. In the present work, six isorhamnetin glycosides (peaks 23–28) were detected, whose mass spectra showed the molecular ion and the fragments due to the loss of sugar units: monoglycosides (ISR-1, ISR-2, ISR-4, ISR-5 and ISR-6) presented the fragment corresponding to the protonated aglycone (isorhamnetin) at *m*/*z* 317 amu; and the diglycoside ISR-3 presented fragments with the same mass differences as quercetin-3-*O*-rhamnoglucoside: a first loss of 146 amu (deoxyhexosyl residue), followed by a loss of 162 amu (hexosyl residue). ISR-2 and ISR-3 were identified as isorhamnetin-3-*O*-glucoside and isorhamnetin-3-*O*-rhamnoglucoside, respectively, by comparison with their corresponding standards.

3.2.4. Hydroxycinnamic acids

5-Caffeoylquinic acid (CA) and 4-*p*-coumaroylquinic acid (PCQ) mass spectra showed their $[M + H]^+$ ions and a fragment corresponding to the cinnamoyl system in the molecule ($[M$-cinnamoyl ion value$]^+$) due to the loss of the esterified quinic acid at *m*/*z* 163 and 147 amu, respectively.

3.2.5. Anthocyanins

In acid media, anthocyanins exist primarily in the coloured form of flavylium cations. Therefore, in their mass spectra in the positive ion mode, the molecular ion ($[M]^+$) and the aglycone ion ($[A]^+$) are observed. Cyanidin-3-*O*-galactoside (ideain) was detected in the apple peel extract by comparison with its standard. Despite its low concentration and the presence of interferences that coeluted, traces of its molecular ion at *m*/*z* 449 amu and of its aglycone ion at *m*/*z* 287 amu were observed.

These results afforded by LC-DAD and LC/APCI-MS provided the molecular mass, the number of hydroxyl groups, the number of sugars and an idea about the substitution pattern of the polyphenols found in the apple peel extract. In order to obtain more precise structural information on the position of the free hydroxyl groups on the polyphenol nucleus, LC-UV with postcolumn addition of shift reagents was performed.

Table 2
Data (*m*/*z*) obtained from the analysis of apple peel extract by HPLC/APCI-MS

Peak	Polyphenol	Code	$[M]^+$	$[M+H]^+$	$[M+H-132]^+$	$[M+H-146]^+$	$[M+H-288]^+$	$[A]^+$	$[A+H]^+$	$[C]^+$[a]
Flavan-3-ols										
1	Procyanidin dimmer	FA-1		579					291	
2	(+)-Catechin	CAT		291						
3	Procyanidin dimmer	FA-2		579					291	
4	Procyanidin B2	PB2		579					291	
5	(−)-Epicatechin	EC		291						
6	Procyanidin trimer	FA-3		867			579		291	
7	Procyanidin dimmer	FA-4		579					291	
8	Procyanidin trimer	FA-5		867			579		291	
9	Procyanidin dimmer	FA-6		579					291	
10	Procyanidin dimmer	FA-7		579					291	
Hydroxycinnamic acids										
11	5-Caffeoylquinic acid	CA		355						163
12	4-*p*-Coumaroylquinic acid	PCQ		339						147
Dihydrochalcones										
13	Hydroxyphloretin diglycoside	PLD-1		585	453				291	
14	Hydroxyphloretin monoglycoside	PLD-2		453					291	
15	Phloretin-2′-*O*-xyloglucoside	PLXG		569	437				275	
16	Phloretin-2′-*O*-glucoside	PLG		437					275	
Flavonols										
17	Quercetin-3-*O*-galactoside	HYP		465					303	
18	Quercetin-3-*O*-glucoside	IQC		465					303	
19	Quercetin-3-*O*-rhamnoglucoside	RUT		611		465			303	
20	Quercetin glycoside (unknown)	QG-C5		435					303	
21	Quercetin-3-*O*-arabinofuranoside	AVI		435					303	
22	Quercetin-3-*O*-ramnoside	QCI		449					303	
23	Isorhamnetin glycoside (unknown)	ISR-1		479					317	
24	Isorhamnetin-3-*O*-glucoside	ISR-2		479					317	
25	Isorhamnetin-3-*O*-ramnoglucoside	ISR-3		625		479			317	
26	Isorhamnetin glycoside (unknown)	ISR-4		449					317	
27	Isorhamnetin glycoside (unknown)	ISR-5		449					317	
28	Isorhamnetin glycoside (unknown)	ISR-6		463					317	
29	Quercetin	QCE		303						
Anthocyanins										
30	Cyanidin-3-*O*-galactoside	IDE	449					287		

[a] $[C]^+$, $[M$-cinnamoyl ion value$]^+$.

3.3. LC-UV photodiode array detection with postcolumn addition of shift reagents

Classical shift reagents for polyphenols have been widely used for the characterisation of phenolic compounds [25,26,28,30]. The crude extract was analysed three times: after postcolumn addition of a weak base, $AlCl_3$ and acidic $AlCl_3$. A weak base (sodium acetate) deprotonates only the more acidic phenolic groups: hydroxyl groups in positions 3, 7 and 4′ (Fig. 1). Aluminium chloride in neutral solution forms complexes with *ortho*-dihydroxyl groups and/or with keto functions having a hydroxyl group in an α- or peri-position. The former complexes are unstable in strong acid solutions. UV band shift interpretation took into account Markham's studies on flavonols, dihydroflavonols and flavanones [28]. Because of structural similarities, band shifts observed in the spectra of hydroxycinnamic acids (containing the cinnamoyl system) were interpreted as for flavonols; and those band shifts in spectra of flavan-3-ols and dihydrochalcones (carbons numbered as shown for catechins in Fig. 1), as for dihydroflavonols and flavanones. Table 3 summarises band shift data for each compound.

3.3.1. UV spectra in the presence of NaOAc

Flavonols (quercetin, quercetin glycosides and isorhamnetin glycosides) (17–29) and 5-caffeoylquinic acid (11) exhibited a bathochromic shift in Band I (33–51 nm), revealing an hydroxyl group in position 4 of the cinnamoyl system. The Band I shift in 4-*p*-coumaroylquinic acid (12) was smaller (11 nm). A bathochromic shift was also observed in Band II of flavonols (33 nm in quercetin, 15 nm in quercetin glycosides, 17–20 nm in isorhamnetin glycosides) as a result of the deprotonation of the 7-hydroxyl group.

With regard to flavan-3-ols and dihydrochalcones, a bathochromic shift of about 12 and 40 nm in Band II, respectively, was detected. The smaller shift observed in flavan-3-ols could be due to the minor conjugation that its structure presents, since it does not contain the keto function in po-

Table 3
Data (*m*/*z*) obtained from the analysis of apple peel extract by HPLC-DAD with and without postcolumn addition of UV shift reagents

Peak	Polyphenol	Code	UV–vis spectra bands (nm)												
			Eluent		NaOAc			$AlCl_3$				Acid $AlCl_3$			
			I	II	Ia	Ib	II	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb
Flavan-3-ols															
1	Procyanidin dimmer	FA-1		279		333	292								
2	(+)-Catechin	CAT		279		343	291								
3	Procyanidin dimmer	FA-2		279		333	291								
4	Procyanidin B2	PB2		279		333	291								
5	(−)-Epicatechin	EC		279		333	291								
6	Procyanidin trimer	FA-3		279		335	291								
7	Procyanidin dimmer	FA-4		279		337	291								
Hydroxycinnamic acids															
11	5-Caffeoylquinic acid	CA	325		372	313*	265	359							
12	4-*p*-Coumaroylquinic acid	PCQ	312		323		293*								
Dihydrochalcones															
13	Hydroxyphloretin diglycoside	PLD-1		282			325								
14	Hydroxyphloretin monoglycoside	PLD-2		286			325								
15	Phloretin-2′-*O*-xyloglucoside	PLXG		286			326								
16	Phloretin-2′-*O*-glucoside	PLG		286			325								
Flavonols															
17	Quercetin-3-*O*-galactoside	HYP	356	256	405	327	271	417	sh	sh	273	sh	353	293*	267
18	Quercetin-3-*O*-glucoside	IQC	355	256	405	329	271	417	sh	sh	273	sh	355	297*	265
19	Quercetin-3-*O*-rhamnoglucoside	RUT	355	256	405	327	271	419	sh	sh	273	sh	355	299*	265
20	Quercetin glycoside (unknown)	QG-C5	356	256	407	327	271	419	sh	sh	273	sh	355	299*	265
21	Quercetin-3-*O*-arabinofuranoside	AVI	354	256	403	327	271	415	sh	sh	273	sh	353	297*	265
22	Quercetin-3-*O*-ramnoside	QCI	350	256	397	329	271	413	sh	sh	273	sh	349		263
23[a]	Isorhamnetin glycoside (unknown)	ISR-1	356	255	401	327	275	391	337	295	265	sh	341	299*	265
24[a]	Isorhamnetin-3-*O*-glucoside	ISR-2													
25	Isorhamnetin-3-*O*-ramnoglucoside	ISR-3	356	256	403	327	275	nd	nd	nd	nd	sh	341	305*	266
26	Isorhamnetin glycoside (unknown)	ISR-4	356	255	395	321	273	389	339	295	275	sh	345	295*	267
27[a]	Isorhamnetin glycoside (unknown)	ISR-5	352	256	393	325	273	387	337	297	271	nd	nd	nd	nd
28[a]	Isorhamnetin glycoside (unknown)	ISR-6													
29	Quercetin	QCE	372	256	405	337	289	429	321	299	269	419	365	299*	263
Anthocyanins															
30	Cyanidin-3-*O*-galactoside	IDE	514	280								529			280

nd, chromatographic peak not detected; sh, shoulder; *, shoulder.
[a] Overlapped chromatographic peaks: 23 and 24, 27 and 28.

sition 4, in contrast to dihydrochalcones, dihydroflavonols and flavanones. Moreover, the 5-hydroxyl group present in the flavan-3-ol structure also contributes to the smaller shift observed in the spectra of this class of polyphenols.

3.3.2. *UV spectra in the presence of $AlCl_3$ and acidic $AlCl_3$*

Flavonol UV spectra in the presence of $AlCl_3$ consist of four absorption peaks: Bands Ia, Ib, IIa and IIb, bathochromically shifted relative to their bands of origin (Band I and II) in the mobile phase spectra. Quercetin glycosides exhibited spectra with bathochromic shifts in Band Ia (57–64 nm) as a result of complexes formed by $AlCl_3$ with 3′,4′-orthodihydroxyl groups in B-ring, and with 4-keto function and 5-hydroxyl group, since 3-hydroxyl group is substituted by a sugar moiety. In acid media, a bathochromic shift persisted in Band I and II relative to the spectra without reagent addition. This fact confirmed the presence of a 5-hydroxyl group. According to Markham [28], Bands Ib and IIa in acidic $AlCl_3$ are shoulders of low intensities. However, in these experiments it was observed that Band Ia was a shoulder of Band Ib, even though the hypsochromic shift of Band Ia of acidic $AlCl_3$ spectra relative to $AlCl_3$ spectra were similar (∼30 nm) to those registered by that author for 3-*O*-glycoside flavonols. In contrast, in the quercetin aglycone spectra, Band Ib was a shoulder of Band Ia, which presented higher intensity, as observed by Markham [28].

UV spectra of isorhamnetin glycosides presented bathochromic shifts of Band I in the presence of $AlCl_3$ (33–35 nm) smaller than those observed in quercetin glycoside spectra. This is due to the fact that in isorhamnetin glycosides, the $AlCl_3$ can only form complexes with the 4-keto function and the 5-hydroxyl group, since they do not contain *ortho*-dihydroxyl groups. In acid media, band shifts in isorhamnetin glycoside spectra were persistent, proving the presence of a 5-hydroxyl group in their structure, since the 3-hydroxyl group was glycosylated. As in quercetin glycosides, Band Ia of isorhamnetin glycosides was a shoulder of Band

Ib. Band Ia in acidic $AlCl_3$ occurred at the same wavelength as Ia in the spectra in $AlCl_3$. This observation confirmed the absence of ortho-dihydroxyl groups in their structures. ISR-3 and the overlapped peaks of ISR-5 and ISR-6 were not detected in the experiments with $AlCl_3$ and acidic $AlCl_3$, respectively; probably due to the fact that they were present in the extract at very low concentrations, near the limit of detection.

As for 5-caffeoylquinic acid UV spectra, a bathochromic shift of Band I (354 nm) was observed due to the complex formed by $AlCl_3$ with 3′,4′-*ortho*-diphenol present in the cinnamoyl system. In acid media, this Band I shift disappeared, which corroborated the presence of *ortho*-dihydroxyl groups. 4-*p*-Coumaroylquinic acid spectrum did not undergo any band shift with this reagent, since it does not contain *ortho*-diphenols in its structure.

Flavan-3-ol and dihydrochalcone UV spectra did not exhibit any band shift in the presence of $AlCl_3$. In these polyphenolic classes, 3′,4′-dihydroxyl groups are not detectable by the addition of this reagent since the B-ring is not conjugated with the major chromophore. On the other hand, these observations also indicated that the A-ring did not contain ortho-dihydroxyl groups. In the presence of acidic $AlCl_3$, UV spectra of dihydrochalcones did not show any bathochromic shift relative to the spectra in the mobile phase. This fact disclosed that the 5-hydroxyl groups in these compounds were substituted.

A bathochromic shift (15 nm) was observed for Band I in the UV spectra of ideain in the presence of acidic $AlCl_3$ as a result of the existence in its structure of 3′,4′-ortho-dihydroxyl groups. This reagent had been already used before for the detection of *ortho*-dihydroxyl groups in anthocyanins [33].

3.4. On-line structure information

LC/APCI-MS and LC-UV together with LC-UV with post-column addition of shift reagents provide a powerful tool for polyphenol analysis in crude plant extracts. These hyphenated techniques give a precise idea of plant constituents, although a complete structural elucidation is not possible in all instances. Regarding the results obtained by the on-line analysis of the Goikoetxea apple peel extract by means of these techniques and by comparing retention times and UV–vis spectra with standards, polyphenols from five classes of phenolic compounds were identified: flavan-3-ols, dihydrochalcones, flavonols, anthocyanins and hydroxycinnamic acids. These results agreed with those obtained by other authors in both dessert apple varieties [10,34] and cider apple varieties [12,13]. Fig. 5 shows examples of the on-line structural information obtained for some polyphenols detected in the apple peel extract.

HPLC-DAD/APCI-MS provided the molecular mass, the number of hydroxyl groups, the number of sugars and an idea about the substitution pattern of polyphenols found in the crude extract. HPLC-DAD with post-column addition of shift reagents gave more precise structural information on the position of the free hydroxyl groups in the polyphenolic nucleus. In this sense, positions of the hydroxyl groups attached to carbons 5, 7, 3′ and 4′ in the structure of quercetin (aglycone) and the six quercetin glycosides detected were confirmed. In accord with their molecular weights, the fragments observed in their mass spectra and their retention times, five glycosides were identified as: quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-rhamnoglucoside (quercetin-3-*O*-rutinoside, rutin), quercetin-3-*O*-arabinofuranoside (avicularin) and quercetin-3-*O*-rhamnoside (quercitrin). A quercetin pentoside (QG-C5) was also detected in the extract. In apple peel, apart from avicularin, other quercetin pentosides have been reported: quercetin-3-*O*-xyloside (reynoutrin) and quercetin-3-*O*-arabinopyranoside (guajaverin) [35,36]. As the chromatographic behaviour of QG-C5 differed notably from that observed for reynoutrin standard, this possibility was therefore, rejected. However, guajaverin standard was not available, and it was not possible to check if it corresponded to QG-C5.

Isorhamnetin and rhamnetin aglycones have the same molecular weight, but are distinguished by the position of their methoxyl group, which is attached to carbon 3′ in the B-ring of isorhamnetin, and to carbon 7 in the A-ring of rhamnetin (Fig. 4). Thus, APCI mass spectra of their glycosides do not contain enough information to differentiate them; since only molecular ions and fragments due to the losses of sugar units leading to the aglycone ion at *m/z* 317 amu are observed. However, band shifts in the UV spectra by post-column addition of UV shift reagents confirmed that the glycosides detected in the extract were isorhamnetin glycosides (ISR-1, ISR-2, ISR-3, ISR-4, ISR-5 and ISR-6). On the one hand, a bathochromic shift of Band II in the presence of NaOAc revealed the existence of a 7-hydroxyl group. On the other hand, no hypsochromic shifts in Band I of UV spectra were observed in acidic $AlCl_3$ media when compared to the spectra in $AlCl_3$ media. This fact implied the absence of *ortho*-hydroxyl groups. The elution order of isorhamnetin glycosides under the analysis conditions was the same as for quercetin glycosides: firstly, those compounds that contained hexoses (ISR-1 and ISR-2) eluted; afterwards, a diglycoside (ISR-3); then, a pentoside (ISR-4); and finally, in the same chromatographic peak, two glycosides were overlapped: one was a pentose (ISR-5) and the other a deoxyhexose (ISR-6). ISR-2

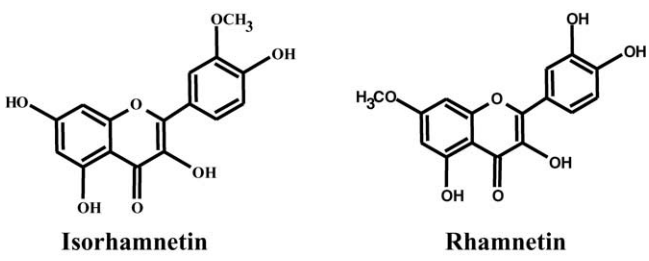

Fig. 4. Structures of isorhamnetin and rhamnetin.

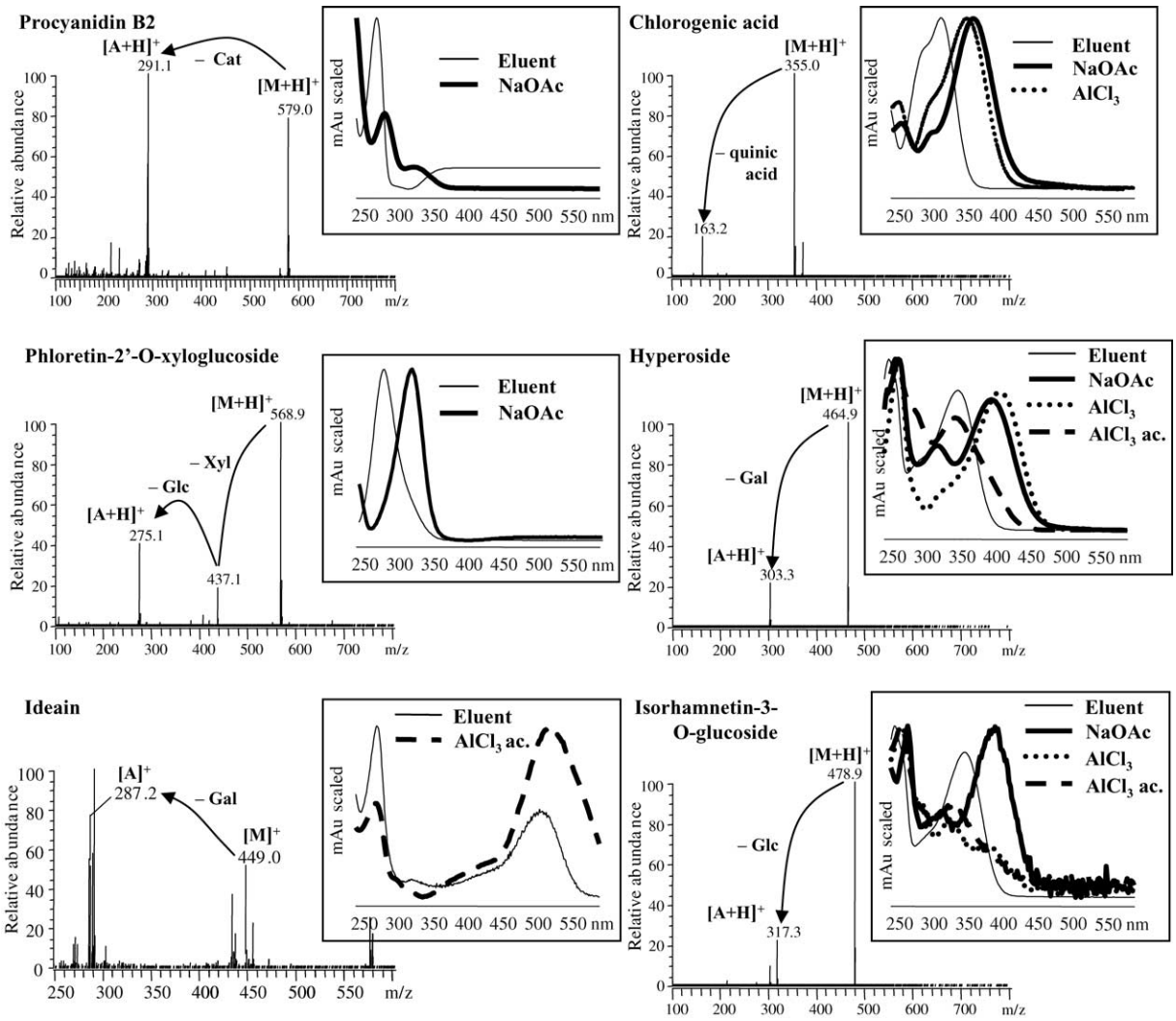

Fig. 5. Examples of on-line structural information obtained for polyphenols present in apple. Abbreviations: Glc, glucose; Gal, galactose; Xyl, xylose; Cat, catechin; ac., acid.

and ISR-3 were identified as isorhamnetin-3-*O*-glucoside and isorhamnetin-3-*O*-rhamnoglucoside, respectively, by comparison with their corresponding standards. Taking into account the similarities of the structure and the elution sequence of isorhamnetin glycosides relative to quercetin glycosides, the former were tentatively identified as: isorhamnetin-3-*O*-galactoside (ISR-1), isorhamnetin-3-*O*-arabinopyranoside (ISR-4), isorhamnetin-3-*O*-arabinofuranoside (ISR-5) and isorhamnetin-3-*O*-rhamnoside (ISR-6).

With regard to hydroxycinnamic acids (5-caffeoylquinic and 4-p-coumaroylquinic acid), the presence of a 4′-hydroxyl group in the cinnamoyl system was confirmed, as well as the 3′,4′-ortho-dihydroxyl group in 5-caffeoylquinic acid. In flavan-3-ols and dihydrochalcones, the presence of 7-hydroxyl groups and the absence of ortho-dihydroxyl groups in the A-ring were proved, disclosing that there could not be free hydroxyl groups in positions 6 and 8. Considering this observation and the conclusion from mass spectral data that the additional hydroxyl group had to be in the polyphenolic nucleus, it could be stated that the additional hydroxyl group in the dihydrochalcones PLD-1 and PLD-2 relative to phloretin-2′-*O*-xyloglucoside and phloretin-2′-*O*-glucoside, had to be found in the B-ring. Although structures have to be confirmed by spectroscopic techniques, it is possible that PLD-1 and PLD-2 were hydroxyphloretin-2′-*O*-xyloglucoside and hydroxyphloretin-2′-*O*-glucoside (traditional numbering of the carbons in dihydrochalcones (Fig. 1)), respectively, with the additional hydroxyl group located at carbon 3 for the following reasons: firstly, data obtained from mass spectra and UV band shifts by shift reagent addition are coherent with the structures proposed. Moreover, in the literature, 3-hydroxyphloridzin has been identified in seeds, pomace and peel from dessert apples [37,38]. Furthermore, during the optimisation procedure for the solid-liquid extraction of apple polyphenols [31], it was observed that the extracted quantities of phloretin-2′-*O*-xyloglucoside and phloretin-2′-*O*-glucoside from apple pulp using acidic methanol were higher than using an aqueous

methanol solvent; in contrast, for PLD-1 and PLD-2, just the opposite occurred, so that, total dihydrochalcone concentrations extracted by both solvents were not significantly different. It seems that methanol reduces polyphenoloxidase (PPO) activity, while solvents with low contents of methanol do not inactivate completely the enzyme [39]. Cresolase activity of PPO is responsible for the enzymatic oxidation of monophenols, such phloretin-2′-*O*-xyloglucoside and phloretin-2′-*O*-glucoside, to ortho-diphenols, generating their corresponding hydroxylated compounds [40,41]. In apple, dihydrochalcones commonly reported have been phloretin-2′-*O*-glucoside (phloridzin) and phloretin-2′-*O*-xyloglucoside. The only bibliographic references about the presence of other dihydrochalcones in apples are those from Foo et al., who detected native 3-hydroxyphloridzin in apple peel and seeds [37,38].

Mass spectral data disclosed that flavan-3-ol dimers (FA-1, FA-2, FA-4, FA-6 and FA-7) and trimers (FA-3 and FA-5) detected in the apple peel extract were only constituted by (+)-catechin and (−)-epicatechin units; being in accordance with the results obtained by other authors [42–44]. Among dimers, procyanidin B2 [(−)-epicatechin (4β → 8) (−)-epicatechin] was identified. However, more precise conclusions on the dimer and trimer structures cannot be achieved with the data obtained from these experiments. In apples, only procyanidins of type B have been found, which are procyanidins that contain just one interflavanic bond C_4–C_6 or C_4–C_8. Certain authors have formally identified some procyanidins, such as dimers B1, B2 and B5; trimers C1 and EC-B5; and tetramers D1 [45,46].

4. Conclusion

The combination of LC/APCI-MS, LC-DAD and LC-DAD with postcolumn addition of UV shift reagents allowed the characterisation of polyphenols previously reported in apple and demonstrated the occurrence of five isorhamnetin glycosides, two hydroxyphloretin glycosides and quercetin in apple peel for the first time. One of these isorhamnetin glycosides was identified as isorhamnetin-3-*O*-rhamnoglucoside. These findings are of great interest for their chemotaxonomic relevance, as well as for the authenticity control of foodstuffs. On the one hand, phloretin derivatives have been generally accepted as apple taxonomic makers. Thus, phloridzin has been used for the detection of fraudulent admixtures. However, phloridzin has been recently identified in strawberry fruits [20]. Other phloretin derivatives such as their xyloglucosides or hydroxyphloretin glycosides could be suitable for authentication purposes, even though an extended screening of polyphenols in strawberry and other fruits is still required in order to clarify whether these dihydrochalcones are present. The phloretin and hydroxyphloretin glycosides have been determined in all Basque cider apple cultivars studied (31 apple varieties) [14,47]. On the other hand, isorhamnetin glycosides detected in apple peel are constituents of pear fruit [21,48,49]. In recent studies, these flavonols have been observed in further apple cultivars: Schieber et al. [22] have found two isorhamnetin glycosides in the Brettacher variety; and the authors have confirmed the occurrence of the six isorhamnetin glycosides studied in three other cider apple cultivars (Patzuloa, Txalaka and Udare Marroi). Hence, both phloridzin and isorhamnetin glycosides are not appropriate to guarantee genuineness of fruit-derived products. These studies contribute largely to the urgent necessity of establishing reliable markers for the authentication of foodstuffs.

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