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Detailed polyphenolic profiling of Annurca apple (M. pumila Miller cv Annurca) by a combination of RP-UHPLC and HILIC, both hyphenated to IT-TOF mass spectrometry

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a b s t r a c t

types of apple, but also several healthy properties.With the aimto thoroughly elucidate the polyphenolic profile of this variety, listed as Protected Geographical Indication product, an extensive qualitative profiling of Annurca apple polyphenolic peel extractwas carried out, by employing a combination of ultra high performance reversed phase (RP-UHPLC) and hydrophilic liquid chromatography (HILIC) coupled to ion trap-time of flight (IT-TOF) mass spectrometry. A total of 63 compounds were tentatively identified, 25 of which not reported in Annurca apple extract so far. Furthermore, thanks to the different selectivity obtained with the HILIC, in combination with accurate mass measurements, an improved separation and detection of procyanidins, was obtained. Moreover, the obtained profiles were compared with those of a conventional variety, such as Red Delicious (RD), highlighting their differences. This work contributes to increase the knowledge about the polyphenolic fingerprint of this typical apple variety.

1. Introduction

Several observations highlight the beneficial effects of polyphenols on human health, such as anticancer (Riboli & Norat, 2003), antioxidant, anti-inflammatory (Pepe et al., 2015) and hypoglycaemic effects (Tenore, Campiglia, Stiuso, Ritieni, & Novellino, 2013). Fruits and vegetables are characterized by high amount of polyphenols. Among widely consumed fruits, apple is one of the most important source of polyphenols in diet, and historically is considered as a "healthy" food, since its regular consumption has been associated with lower onset of cardiovascular diseases (Knekt, Jarvinen, Reunanen, & Maatela, 1996) and different types of cancer (Eberhardt, Lee, & Liu, 2000). Apples contain a wide variety of polyphenolic classes: hydroxycinnamic acids, dihydrochalcones, flavonols, anthocyanins, and flavan-3-ols. Usually the peel contains the highest concentration of these bioactive compounds, with respect to flesh and core (Kalinowska, Bielawska, Lewandowska-Siwkiewicz, Priebe, & Lewandowski, 2014). Within the different classes of apples, "Annurca" is a typical cultivar of Southern Italy, in particular of Campania region, and has been listed as a Protected Geographical Indication (IGP) product from the European Council [Commission Regulation (EC) No. 417/2006]. It is characterized from a rispy flesh and a fragrant flavor (Fratianni, Sada, Cipriano, Masucci, & Nazzaro, 2007), the high acid/sugar ratio gives a different taste from other types of apples. This cultivar is subjected to a particular reddening treatment, with controlled exposure to sun and temperature (D'Abrosca, Fiorentino,Monaco, Oriano, & Pacifico, 2006).Many biological activities have been reported for the "Annurca" polyphenolic extract, such as: antioxidant (Napolitano et al., 2004), anticancer (Fini et al., 2007) and hypoglycaemic (Tenore, Campiglia,

Ritieni, & Novellino, 2013). In order to explain biological activity by structure–activity relationship, it is important to possess a deep knowledge of the polyphenolic profile in this matrix, as well as in nutraceuticals based on apple extracts. Regarding the composition of Annurca apple, only few analytical methods have been reported, mainly carried out by high performance liquid chromatography (HPLC) (Mari et al., 2010; Panzella, Petriccione, Rega, Scortichini, & Napolitano, 2013) employing C18 columns packed with conventional fully porous particles, coupled to low resolution mass spectrometers, such as ion trap (IT) and triple quadrupole (QqQ), and recently ultra high performance liquid chromatography (UHPLC)with diode array detector (DAD) (Fratianni, Coppola, & Nazzaro, 2011). Furthermore, in this matrix, particularly challenging is the separation of condensed tannins or proanthocyanidins, a class of phenols composed of flavan-3-ol monomeric units joined through interflavanoid linkages, divided into various subclasses with procyanidins, based on (epi)catechin units, and prodelphinidins, comprising (epi)gallocatechin units. Monomers are frequently linked through C4 → C6 or C4 → C8 bonds (B-type), or, more rarely, a second bond can occur fromoxidative coupling of C2→O7 to form A-type oligomers.

This class of compounds, especially those with high degree of polymerization (DP), tends to co-elute in reversed phase liquid chromatography (RP-LC) in an unresolved "hump" (Lazarus, Adamson, Hammerstone, & Schmitz, 1999) which hinders their resolution and detection. Although UHPLC conditions, using sub-2 μm particle C18 columns, improve the resolution of proanthocyanidins (Kalili, Cabooter, Desmet, & de Villiers, 2012), the separation of complex mixtures, containing both polymerized procyanidins and other polyphenolic molecules, still remains a challenge. A valid option, for procyanidin separation, is represented by normal phase liquid chromatography (NP-LC) (Lazarus et al., 1999), and recently, by hydrophilic liquid chromatography (HILIC) as reported for cocoa procyanidins (Kalili & de Villiers, 2009; Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006). In HILIC, a polar stationary phase is used in combination with aqueous mobile phase in order to separate analytes according to polarity. The probable retention mechanisminvolves partitioning of analytes between the mobile phase and a water-layer immobilized on the stationary phase (Nguyen & Schug, 2008). In this work with the aim to elucidate in detail the qualitative profiling of Annurca apple polyphenols, we developed a combined approach, based on ultra high performance reversed phase (RP-UHPLC) and HILIC. Both separation techniques were hyphenated with a hybrid ion trap-time of flight (ITTOF) mass spectrometer. The polyphenolic profiles were compared with those of Red Delicious variety, highlighting their differences. The developed method contributes to improve the knowledge about this typical variety and represents a valid tool for the comparison with other apple extracts.

2. Materials and methods

2.1. Chemicals

Ultra purewater (H_2O)was obtained by aMilli-Q system(Millipore, Milan, Italy). The following chemicals have been all purchased from Sigma Aldrich (Milan, Italy): acetonitrile (ACN), and acetic acid LC–MS grade (CH_3COOH), methanol (CH_3OH) and formic acid (HCOOH). Standards of phloridzin, cyanidin-3-O-galactoside, quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-xyloside and isorhamnetin-3-O-glucoside were purchased from ExtraSynthese (Lione, France).

Standards of (+)-catechin and (−)-epicatechin were purchased from Sigma Aldrich.

2.2. Fruit collection and sample preparation

Annurca (Malus pumila Miller cv Annurca) and Red Delicious (M. pumila Miller cv Red Delicious) apple fruits were acquired in a local store in Fisciano (SA, Campania, Italy). Fresh peels (10 g) of apple samples were homogenized by using an IKA Ultra-Turrax T-25 tissue homogenizer (IKA works Inc., Wilmington, NC, USA) and extracted in 30 mL methanol/water (80:20) with 0.1% v/v HCOOH for 1 h at room temperature

to extract phenolic compounds. The mixture was centrifuged and the supernatants collected and filtered through 0.45 μm nylon membrane filters and injected for LC–MS analysis.

2.3. Instrumentation

RP-UHPLC and HILIC analyses were both performed on a Shimadzu Nexera UHPLC system (Shimadzu, Milano, Italy), consisting of a CBM-20A controller, four LC-30AD dual-plunger parallel-flow pumps, a DGU-20 A5 degasser, an SPD-M20A photo diode array detector (equipped with a semi-micro flow cell of 2.5 μL), a CTO-20A column oven, and a SIL-30AC autosampler. The UHPLC system was coupled online to an LCMS–IT-TOF hybrid mass spectrometer through an ESI source (Shimadzu, Kyoto, Japan). LC–MS data elaborationwas performed by the LCMSsolution® software (Version 3.50.346, Shimadzu).

2.4. RP-UHPLC and HILIC conditions

Two different columnswere employed in thiswork: a Kinetex™C18 150 mm × 2.1 mm, 2.6 μm (Phenomenex®, Bologna, Italy) for the RPUHPLC analysis, and a Luna® HILIC 150 mm × 2.0 mm, 3.0 μm (Phenomenex®), for the HILIC analysis. For RP-UHPLC analyses the mobile phase employed was (A): 0.1% CH3COOH in water v/v, (B) 0.1% CH3COOH in ACN v/v, and analysis was performed in gradient elution as follows: 0–5 min, 0–10% B, 5–20 min, 10–20% B, 20–25 min, 20–50% B, 25–26 min, 50–70% B, 26–27 min, 70–95% B. Flow rate was set at 0.5 mL/min. Column oven temperature was set to 40 °C. 2 μL of Annurca extract was injected. For the HILIC analyses the mobile phase employed was: (A) 0.1% CH3COOH in water/ACN: 80W/20ACN, (B) CAN plus 0.1% CH3COOH, analysis was performed in gradient elution as follows: 0–4min, isocratic at 100% B, 4–60 min, and 100–40% B. Column oven temperature was set to 25 °C. 1 μL of extract was injected. The following photo diode array (PDA) detector parameters were applied: sampling rate, 12.5 Hz; detector time constant, 0.160 s; and cell temperature, 40 °C. Data acquisition was set in the range 190–500 nm and chromatograms were monitored at 280 and 320 nm at the maximum absorbance of the compounds of interest.

2.4.1. LCMS–IT-TOF parameters

UHPLC systemwas coupled on-line to a hybrid IT-TOF instrument, in RP-UHPLC flow rate from LC was split 50:50 prior of the electrospray (ESI) source by means of a stainless steel tee union (1/16 in., 0.15 mm bore, Valco HX, Texas U.S.). The IT-TOF analyzer was tuned using a standard sample solution of sodium trifluoroacetate. MS detection was operated in negative ionization mode with the following parameters: detector voltage: 1.57 kV, interface voltage:−3.5 kV, curve desolvation line (CDL) temperature: 250 °C, block heater temperature: 200 °C, nebulizing gas flow (N2): 1.5 L/min, and drying gas pressure: 100 kPa.

Full scan MS data were acquired in the range of 200–1600 m/z, ion accumulation time: 25 ms, ion trap repeat: 3. MS/MS experiments were conducted in data dependent acquisition, precursor ions were acquired in the range 150–1600 m/z, peak width, 3 Da, ion accumulation time: 50ms, collision induced dissociation (CID) energy: 50%, collision gas: 50%, ion trap repeat: 1, execution trigger (BPC) intensity at 95% top level. For the prediction ofmolecular formula the “Formula Predictor” software (Shimadzu) was used with the following settings: maximum deviation from mass accuracy: 5 ppm, fragment ion information, and nitrogen rule.

3. Results and discussion

3.1. RP-UHPLC–ESI–IT-TOF profiling of polyphenolic extracts As can be observed fromFigs. 1 and S1, showing the UV profile of the Annurca and R.D extract recorded at 280 nm, the entire separation was completed in less than 25 min, no peaks were observed after this time.

Several parameters were investigated to obtain satisfactory resolution, such as flow rate, temperature, and mobile phases (Figure S8). A flow rate of 0.5 mL/min and a column temperature of 40 °C gave the best results in terms of analysis time and peak overlap. Acetonitrile was used as organic modifier instead of methanol, resulting in lower backpressure, and acetic acid provided the better ionization efficiency compared to formic (data not shown).

MS ionization was operated in negative mode, since it provided the highest sensitivity. The identification of compounds was based on accurate MS and MS/MS spectra, retention time of available standards, and comparison with literature.
The following free on-line databases were consulted: ChemSpider (http://www.chemspider.com), SciFinder Scholar (https://scifinder. cas.org) and Phenol-Explorer (www.phenol-explorer.eu). The list of tentatively identified compounds by RP-UHPLC–MS is reported in Table 1 in order of class.

3.1.1. Hydroxycinnamic acids
Hydroxycinnamic acids eluted from 4 to 7.5 min, the MS/MS spectrum of first eluting compound (rt 4.45) revealed the loss of a hexose [M-H-162]−, thus was characterized as 4-hydroxybenzoic acid-4-O-hexoside (De la Luz Cádiz-Gurrea, Fernández-Arroyo, Joven, & Segura-Carretero, 2013). Compounds 2 and 15 (rt 5.16, 7.34) were both characterized by MS/MS fragments at m/z 191.0571, of the deprotonated quinic acid moiety, and m/z 163.0348 [quinic acid-HCO]−, and were proposed as 4 and 5-p-coumaroylquinic acid respectively (Fromm, Loos, Bayha, Carle, & Kammerer, 2013). Also characterized from the loss of quinic acid and from fragment at m/z 173.0400 [quinic acid-H-H2O]−, were compounds 6 and 12 (rt 5.97, 6.94), so they were tentatively assigned as 5′ caffeoylquinic acid (chlorogenic acid) and its 3′ isomer (Ramirez-Ambrosi et al., 2013). A fragment at m/z 163.0353 was observed in the MS/MS spectrum of compound 8 (rt 6.28), as probable result of a hexose loss, and was proposed as 5-pcoumaroyl-hexoside (Sanchez-Rabaneda et al., 2004). Compounds 1–2–15–8 were not reported in previous investigations in Annurca extracts (Lamperi et al., 2008; Mari et al., 2010).

3.1.2. Dihydrochalcones
Compound 34 (rt 12.36) showedMS/MS fragments at m/z 289.0695 and 271.0571, the first deriving fromthe consequential loss of a pentose and a hexose moiety, while the second denotes the possible loss of an hydroxyl group, and was tentatively assigned as 3-hydroxyphloretin-2-O-xylosyl-glucoside, this observation is in accordance with previous MS data on apple extracts (Alonso-Salces et al., 2004; Ramirez-Ambrosi et al., 2013). Peak 39 (rt 13.77) presented a MS/MS fragment at m/z 273.0742, resulting from the loss of two hexose moieties, of the deprotonated aglycone phloretin (C15H14O5), andwas tentatively recognized as phloretin-di-hexoside (Fromm et al., 2013). Peak 47 (rt 15.31) exhibited an intense MS signal and absorbance at 280 nm, the m/z at 273.0748 was the dominant fragment in MS/MS spectrum, generated as for peak 34, from the cleavage of two sugar moieties, and was tentatively characterized as phloretin-2′-O-xylosylglucoside.
The same fragmentation pattern was observed for peak 50 (rt 15.72), which was assigned as phloretin-pentosyl-hexoside, other complementary techniques are necessary to confirm this hypothesis, in accordance with previous literature (Ramirez-Ambrosi et al., 2013; Reis, Rai, & Abu-Ghannam, 2012). Last compounds of this class, 55 + 56, co-eluted in an intense peak (rt 17.39), the compound with [M-H]− 435.1311, was easily identified as phloridzin by comparison with standard rt, the loss of 162 amu highlights the presence of glucose, this compound represents one of the most abundant compounds in apples (Fromm, Bayha, Carle, & Kammerer, 2012). The co-eluted compound, with [M-H]− 549.1222, shows both the phloridzin m/z 435.1309 (C21H24O10) and the phloretin m/z 273.0723 signals in the MS/MS spectrumas can be observed in Figure S2, thereafter could probably be a phloridzin derivate, NMR and MSn experiments are necessary to confirm the structure. In this class peaks 34, 39 and 56 were not reported in Annurca, to the best of our knowledge (Lamperi et al., 2008; Mari et al., 2010).

3.1.3. Anthocyanins

One anthocyanin was detected, even if themobile phase pH was not as low as the optimal for the separation of these compounds, furthermore the absorbance at 500 nm was weak, indicating a low concentration (Garcia-Beneytez, Cabello, & Revilla, 2003). Peak 3 (rt 5.27,) showed a typical ionization profile of anthocyanins in negative mode (Figure S3), in the full scan depicted, ions at m/z 465.1055 and 447.0977 correspond to the adduct [M-2H + H2O]− and to [M-2H]−(Sun, Lin, & Chen, 2012). The fragment ion at m/z 285.0392 [M-2H-162]− represents the deprotonated aglycone cyanidin (C15H11O6), and assumes the loss of a hexose, finally leading, by additional comparison with standard rt, to its identification as cyanidin-3-O-galactoside.

3.1.4. Flavonols

A large number of flavonol glycosides were detected, which can be classified into quercetin, isorhamnetin and kaempferol derivatives.

3.1.4.1. Quercetin derivatives. Compounds 30 and 31 (rt 11.23, 11.44) having [M-H]− at m/z 595.1308 possessed the same fragment ion at m/z 301.0324 with molecular formula C15H10O7, probably derived fromthe sequential loss of a pentose and a hexose, theywere tentatively assigned as quercetin-3-O-pentosyl-hexoside derivatives (Oszmianski, Wojdylo, Gorzelany, & Kapusta, 2011). Peak 35 (rt 12.75) was the most intense in the profile, his fragment ion, again at m/z 301.0338, points out the quercetin aglycone, and is the same observed for compound 38. By comparison with the standard rt, these compounds were identified as quercetin-3-O-galactoside and quercetin-3-Oglucoside respectively, with the galactoside form that elutes first (Figure S4), as observed in literature (Kalili & de Villiers, 2009). Peak 36 (rt 12.93), was characterized by an ion at m/z 463.0884 derived from an in source fragmentation and, in the MS/MS spectrum, a fragment at m/z 301.0335which suggests the loss of a rhamnose and a hexose, and was identified as rutin (Sommella et al., 2013). An in source fragmentation occurred also for peak 41 (rt 13.99), with signals at m/z 505.0991 and 463.0874, and MS/MS fragments at m/z 505.0990 and 301.0245. The mass difference suggests the loss of a malonyl residue followed by loss of a hexose, the proposed structure is quercetin 3-O-(6″-malonyl-hexoside), which needs confirmation by further experiments.

Peaks 43–44–46 (rt 14.25, 14.44, 15.18) were all characterized by the same mass and fragmentation [M-H-132] −, hence, based on a previous observation, regarding the elution order of quercetin glycosides in reversed phase (Schieber, Conrad, Beifuss, & Carle, 2002) and considering the retention time of quercetin-3-O-xyloside standard, they were assigned as quercetin-3-O-xyloside, 3-O-arabinopyranoside, and 3-O-arabinofuranoside respectively. Peak 45 (rt 14.78) gave two MS/MS signals at 463.0893 and 301.0326 m/z, the mass difference could refer to the loss of an acetate group [M-H-42]−, and a hexose respectively, the proposed identification is quercetin-3-O-hexosyl-6″-acetate,which needs to be confirmed. A complex structurewas hypothesized for peak 48 (rt 15.49) (Figure S5). The ion atm/z 463.0867 can be attributed to the loss of a methylglutaryl moiety [M-H-144] −, while the ion at m/z 505.0997 to a possible rearrangement into a 6″ acetate form, these information led to the tentative assignment as quercetin-3-O-[6″-(3-hydroxy-3-methylglutaryl)]-β-hexoside, a similar structure has been reported recently (Porter, Van den Bos, Kite, Veitch, & Simmonds, 2012). The difference of 132 Da with the fragment ion at m/z 301.0336, suggests a pentose loss for peak 49 (rt15.62), that was tentatively identified as quercetin-3-O-pentoside (Ramirez-Ambrosi et al., 2013). Peak 51 (rt 15.96), withMS/MS 301.0327, showed a difference of 146 Da, corresponding to the loss of rhamnose, and leading to possible identification as quercetin-3-O-rhamnoside. As Table 1 summarizes among quercetin derivatives five compounds have been reported for the first time in this variety.

3.1.4.2. Isorhamnetin derivatives.
Peaks 52 and 54 (rt 16.22, 16.81) exhibited the same precursor ion, and their main fragment ions, at m/z 315.0488, with molecular formula C16H12O7, belong to the deprotonated aglycone isorhamnetin, hence, by comparison with the retention time of isorhamnetin-3-O-glucoside standard they were finally tentatively identified as 3-O-galactoside and 3-O-glucoside forms respectively (Schieber, Keller, Streker, Klaiber, & Carle, 2002). Peak 53 (rt 16.44) showed, in a similar manner to rutin, the loss of two sugar moieties, [M-H-146-162]–, identifying the compound as isorhamnetin-3-O-rutinoside in accordance with previous Q-TOF data (Ramirez-Ambrosi et al., 2013). MS/MS spectra of peaks 59 and 61 (rt 18.53, 19.44) revealed a similar structure to compound 51,with a loss of methylglutaryl [M-H-144]– group, but, in this case the ion at m/z 315.0494 suggested the isorhamnetin aglycone, thus they were proposed as isorhamnetin-3-O-[6″-(3-hydroxy-3-methylglutaryl)]-β-hexoside derivatives (Figure S6), as for peak 48, complementary techniques are mandatory to confirm these structures. Peaks 60 and 62 (rt 18.97, 19.60) showed similar fragmentation pattern, with MS/MS fragment ion at m/z 315.0503 and 315.0271 respectively, the difference of 132 Da suggests the loss of a pentose moiety, leading to their tentative identification as isorhamnetin-3-O-pentosides. As for peak 54, the difference of 146 Da points out the loss of rhamnose, and the last eluting peak, 63 (rt 20.22), was finally identified as isorhamnetin-3-Orhamnoside (Alonso-Salces et al., 2004). Only one isorhamnetin derivative was reported in Annurca extract (Mari et al., 2010).
3.1.4.3. Kaempferol derivatives. Peak 57 (rt 18.07) showed MS/MS fragment ions at m/z 285.0839 and 255.0272, the difference of 204 Da suggests the loss of a hexose, and an acetyl moiety, as already reported previously (Karthivashan, Tangestani Fard, Arulselvan, Abas, & Fakurazi, 2013). This was proposed as kaempferol-3-O-(6″-O-acetyl)-hexoside, and was detected only in Annurca extract. Peak 58 (rt18.18) showed the same fragmentation pattern, the loss of 132 Da revealed the presence of a pentose moiety, this was tentatively identified as kaempferol-3-O-pentoside, in accordance with accurate MSn data (March & Miao, 2004).

3.1.5. Flavanones
One flavanone-glycoside was detected, peak 32 (rt 11.78), the main fragment ion, at m/z 271.0611, is probably due to the loss of a hexose (162 Da), by comparison with previous MS/MS data on apple (Sanchez-Rabaneda et al., 2004) pomace, this compoundwas characterized as naringenin-O-hexoside, and was not reported so far in Annurca extract.
3.1.6. Flavan-3-ols, Peaks 7 and 16 (rt 6.01, 7.46), possessing the same parent and fragment ions, were identified by further comparison with the corresponding standards, as (+)-catechin and (−)-epicatechin respectively.
MS/MS spectrum of peak 4 (rt 5.45) was characterized by fragments at m/z 289.0705 and 245.0782, the loss of 162 Da can be attributed to a hexose moiety, thus the compound was tentatively identified as catechin-3-O-hexoside. The fragmentation pattern of peak 42 (rt 14.08) was similar to peak 4, revealing a possible (epi)catechin structure, this compound was tentatively identified as unknown catechin- 3-O-hexoside derivative. In this class only peaks 7 and 16 have already been reported previously in Annurca.

3.1.7. Procyanidins
Multiple isomers were detected, in particular: four dimeric, five trimeric, five tetrameric, five pentameric, two hexameric and one heptameric isomers were detected. Peaks 5–13–25–37 (rt 5.73, 7.11, 9.51, 13.14) showed similar fragmentation pattern, ions with m/z 289.0767 belong to the monomer (epi)catechin as consequence of
quinone methide (QM) cleavage of the interflavan bond, while fragments at 425.0869 and 407.0754 corresponding to a retro-Diels– Alder (RDA) mechanism [M-H-152]– and subsequent

loss of water respectively. Based on these informations thesewere tentatively identified as (epi)catechin dimers (Gu et al., 2003). Peaks 9–14–21–27–40 (rt 6.45, 7.22, 8.98, 10.29, 13.92) were all characterized by the fragment at m/z 739.1626, probably resulting from the loss of phloroglucinol unit (heterocyclic ring fission, HRF, −126 Da), and other fragments such as m/z 577 and 289 as a result of loss of (epi)catechin units, referring on previous MS data (Montero, Herrero, Ibáñez, & Cifuentes, 2013) these compounds were characterized as (epi)catechin trimers. Likewise, peaks 10–11–18–19–26 (rt 6.64, 6.77, 7.60, 7.86, 9.91) were identified as (epi)catechin tetramers. Fragmentation pattern of peaks 17–20–23–28–29 (rt 7.59–8.83–9.40–10.44–10.99) were characterized by multiple loss of 289 Da, resulting from consecutive (QM) cleavages between the flavan units, according to previous Q-TOF data on apple procyanidins (Montero, Herrero, Ibáñez, & Cifuentes, 2013) these compounds were proposed as (epi)catechin pentamers. In a similar manner, peaks 22–33–24 (rt 9.37, 12.28, 9.41)were tentatively assigned as (epi)-catechin hexamers and heptamers respectively. Parent ions from tetramers to heptamer were all detected as doubly charged [M-2H]2–.
As can be observed fromretention times in Table 1, the elution order of oligomeric procyanidins in reversed phase is not related onmolecular mass (Kalili & de Villiers, 2009). Since some compounds of this class are not fully resolved in these conditions, and often coelute with resulting decreased ionization efficiency, a different separation mechanism was investigated in this study.

3.2. Comparison with Red Delicious extract polyphenolic profile

Among 63 identified compounds in Annurca extract, 13 of them were not detected in Red Delicious extract. In particular, for dihydrochalcones compound 39 phloretin-di-hexoside was not revealed in the R.D extract. Regarding quercetin glycosides compounds 30 and 31, proposed as pentosyl hexosideswere absent, aswell as peaks 45 and 48 quercetin-3-O-hexosyl-6″-acetate quercetin-3-O-[6″-(3-hydroxy-3-methylglutaryl)]-β-hexoside were not detected in R.D extract. It is noteworthy that the presence of only two isorhamnetin derivatives was found in R.D extract, namely compounds 52 and 54,whereas, as can be clearly observed from chromatogram comparison of the extract (Fig. 2), the marked isorhamnetin derivatives were detected only in the Annurca extract.

3.3. Improving separation and detection of procyanidins by HILIC–IT-TOF–MS

In the last years HILIC chromatography has been proposed as a valid alternative to RP for procyanidin separation (Karonen, Liimatainen, & Sinkkonen, 2011). A variety of HILIC stationary phases is commercially available, an important application of this techniquewas the separation of cocoa and apple (Red Stark variety) procyanidins by off-line comprehensive two dimensional chromatography (Kalili & de Villiers, 2009), in which the authors used a diol stationary phase in the first dimension. In thisworkwe employed a HILIC column with a cross-linked diol stationary phase. As well as for RP-UHPLC, several parameters, reported in Section 2.4, were optimized in order to reach a satisfactory resolution.
The UV–HILIC chromatogram is reported in Fig. 3. Non-procyanidin compound, like flavonols, hydroxycinnamic acids and dihydrochalcones were not well retained under these conditions even with a short isocratic initial step, and tend to elute earlier in broad peaks (data not shown). In contrast, an efficient separation of procyanidins was obtained, sharper peakswere obtainedwith respect to previous normal-phase separations which used non-aqueous solvents. It is clearly visible that procyanidins are separated according to increasing molecular weight, and hence DP, under these conditions. In this separation, each DP possesses multiple peaks or shoulders as visible in Fig. 3, representing compounds with the same molecular mass but different connectivities, such as regio- or stereo-isomers, as previously reported also in NP

conditions (Robbins et al., 2009). As can be observed from Table 2, singly charged molecular ions [M-H]− were detected for oligomers with degree of polymerization (DP) 2 to 4, whereas higher oligomers were detected as doubly charged ions [M-2H]2−.With respect to the RP analysis, the separation and detection, by ESI–MS, of oligomers starting from degree of polymerization four (DP 4) is enhanced. In particular, the detection of oligomers with DP 6 and 7 was significantly improved.

From the MS spectra in Fig. 4a,b, it can be appreciated how hexamer signals were covered by co-elutions in RP-UHPLC–MS (Fig. 4a), but fully resolved and clearly detected with higher intensity in the HILIC– MS (Fig. 4b). This is not only due to a better separation of these analytes, but also to the gain in ionization efficiency, since the highly organic mobile phase used enhanced the ESI sensitivity (Nguyen & Schug, 2008). In this regard the presence of oligomers above DP 6 has been only mentioned in Annurca (Lamperi et al., 2008). Figure S7 shows MS and MS/MS of a compound not detected by RP-UHPLC–MS. Fragments at m/z 1008.2047, 863.1777 and 575.1144 could result from HRF and fromthe consecutive QMcleavage of flavan units respectively, whereas fragments at m/z 737.1054 and 449.0860 are the products of a phloroglucinol loss (126 Da) from fragments at m/z 863 and 575 respectively. By these information, together with Orbitrap–MS spectra comparison in literature (Lin, Sun, Chen, Monagas, & Harnly, 2014), these compounds were tentatively identified as epi(catechin) octamers (DP 8). Increasing the retention, the resolution and peak areas decrease (Kelm et al., 2006) and after oligomers with DP 8, very broad and small peakswere observed. TwoMS signalswere detected, even if their intensity was very low, which could be addressed to the possible presence of oligomers with DP 9–10, further experiments are necessary to confirm these data. Only B type procyanidins were detected in this variety of Annurca, and the same profile was obtained for the RD extract.

4. Conclusions

The employment of two different separation techniques, such as RPUHPLC and HILIC, in combination with a hybrid IT-TOF mass spectrometer, led to the tentative identification of 63 compounds, 25 not previously reported in Annurca apple, which represents an improvement with respect to the previous observations. Employing two different separation methods and accurate mass measurements, enhances the separation and detection of every polyphenolic class, reporting the presence of compounds not previously detected in this apple kind by conventional HPLC–MS based techniques. RP-UHPLC–MS was suitable for the analysis of dihydrochalcones, hydroxycinnamic acids, flavonols and anthocyanins, but not satisfactory for procyanidins. In this regard the employment of HILIC–MS improved the separation and detection of oligomeric procyanidins which are very interesting molecules for human health, but often overlooked (Cos et al., 2004), thus it is important to extend the knowledge about their natural sources, even if their concentration can be low. As a result, the presence of oligomers with DP N 6 was described in detail for the first time in this apple variety. Moreover 13 compounds were not detected in Red Delicious extract.

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Table 1
List of polyphenolic compounds detected by RP-UHPLC–ESI–IT-TOF.

Peak	t_r	Molecular formula	[M-H]$^-$	[MS/MS]	PDA (nm)	Error (ppm)	Compound	References
Hydroxycinnamic acids								
1	4.45	$C_{13}H_{16}O_8$	299.0758	173.0735	320	−4.68	4-Hydroxybenzoic acid-4-*O*-hexoside**	De la Luz Cádiz-Gurrea et al., 2013
2	5.16	$C_{16}H_{18}O_8$	337.1122	191.0571 [Quinic acid-H]$^-$ 163.0348 [Quinic acid-H-CO]$^-$	312	−5.34	4-*p*-Coumaroylquinic acid**	Fromm et al., 2013
6	5.97	$C_{16}H_{18}O_9$	353.0873	191.0569 191.0571 [Quinic acid-H]$^-$ 173.0400 191.0571 [Quinic acid-H-H_2O]$^-$	324	−0.85	5′- Caffeolquinic acid (Chlorogenic Acid)	Ramirez-Ambrosi et al., 2013
8	6.28	$C_{15}H_{18}O_8$	325.0924	163.0353 [Coumaroyl acid-H]$^-$ 191.0571	315	−1.54	5-*p*-Coumaroyl hexoside**	Sanchez-Rabaneda et al., 2004
12	6.94	$C_{16}H_{18}O_9$	353.0846	191.0570 [Quinic acid-H]$^-$ 173.0447 [Quinic acid-H-H_2O]$^-$	324	−4.16	3′- Caffeolquinic acid	Ramirez-Ambrosi et al., 2013
15	7.34	$C_{16}H_{18}O_8$	337.0942	191.0588 [Quinic acid-H]$^-$ 163.0441 [Quinic acid-H-CO]$^-$	312	−1.19	5-*p*-Cumaroylquinic acid b**	Fromm et al., 2013
Dyihydrochalcones								
34	12.39	$C_{26}H_{32}O_{15}$	583.1652	289.0695 [Y_0]$^-$ 271.0571 [Y_0-H2O]$^-$	285	−2.23	3-Hydroxyphloretin-2-*O*-xylosyl-glucoside**	Ramirez-Ambrosi et al., 2013
39	13.77	$C_{27}H_{34}O_{15}$	597.1802	273.0742 [Y_0]$^-$	284	−1.51	Phloretin-di-hexoside**,#	Fromm et al., 2013
47	15.31	$C_{26}H_{32}O_{14}$	567.1738	273.0748 [Y_0]$^-$	285	1.94	Phloretin-2′-*O*-xylosyl-glucoside	Ramirez-Ambrosi et al., 2013
50	15.72	$C_{26}H_{32}O_{14}$	567.1736	273.0745 [Y_0]$^-$	285	1.94	Phloretin-pentosyl-hexoside	Ramirez-Ambrosi et al., 2013
55	17.39	$C_{21}H_{24}O_{10}$	435.1311	273.0751 [Y_0]$^-$ 167.0375 [$C_8H_7O_4$]$^-$	284	2.76	Phloridzin	Fromm et al., 2013
56	17.48	$C_{25}H_{26}O_{14}$	549.1222	435.1309 [$C_{21}H_{24}O_{10}$]$^-$ 273.0723 [$C_{15}H_{14}O_5$]$^-$	282	−5.10	Unknown phloridzin derivate **	
Anthocyanins								
3	5.27	$C_{21}H_{22}O_{12}$	465.1055	285.0392 [Y_0-2H]$^-$ 241.0502 199.0416	500	3.66	Cyanidin-3-*O*-galactoside	Garcia-Beneytez et al., 2003
Flavonols								
30	11.23	$C_{26}H_{28}O_{16}$	595.1308	301.0324 [Y_0]$^-$ 271.0227 [Y_0-CHO]$^-$ 255.0282 [Y_0-CHO-OH]$^-$	269	0.50	Quercetin-3-*O*-pentosyl hexoside**,#	Oszmianski et al., 2011
31	11.44	$C_{26}H_{28}O_{16}$	595.1297	301.0319 [Y_0]$^-$ 271.0237 [Y_0-CHO]$^-$ 255.0294 [Y_0-CHO-OH]$^-$	269	−1.34	Quercetin-3-*O*-pentosyl hexoside**,#	Oszmianski et al., 2011
35	12.75	$C_{21}H_{20}O_{11}$	463.0897	301.0338 [Y_0]$^-$ 271.0242 [Y_0-CHO]$^-$	255	3.24	Quercetin-3-*O*-galactoside (Hyperoside)	Kalili & de Villiers, 2009
36	12.93	$C_{27}H_{30}O_{16}$	609.1465	301.0335 [Y_0]$^-$ 271.0237 [Y_0-CHO]$^-$	353	−1.15	Rutin	Sommella et al., 2013
38	13.29	$C_{21}H_{20}O_{11}$	463.0890	301.0336 [Y_0]$^-$ 271.0241 [Y_0-CHO]$^-$	255	1.73	Quercetin-3-*O*-glucoside (Isoquercetin)	Kalili & de Villiers, 2009
41	13.99	$C_{24}H_{22}O_{15}$	549.0884	505.0990 [COO]$^-$ 301.0245 [Y_0]$^-$	329	−0.36	Quercetin-3-*O*-(6″-malonyl-hexoside)**	Schieber, Conrad, Beifuss, & Carle, 2002; Schieber, Keller, Streker, Klaiber, & Carle, 2002
43	14.25	$C_{20}H_{18}O_{11}$	433.0773	301.0338 [Y_0]$^-$ 271.0246 [Y_0-CHO]$^-$	353	1.85	Quercetin-3-*O*-xyloside (Reynoutrin)	Schieber et al., 2002
44	14.44	$C_{20}H_{18}O_{11}$	433.0770	301.0335 [Y_0]$^-$ 271.0249 [Y_0-CHO]$^-$	255	1.88	Quercetin-3-*O*-arabinopyranoside (Guajaverin)	Schieber, Conrad, Beifuss, & Carle, 2002; Schieber, Keller, Streker, Klaiber, & Carle, 2002
45	14.78	$C_{23}H_{22}O_{13}$	505.1003	463.0893 301.0326 [Y_0]$^-$ 271.0239 [Y_0-CHO]$^-$	353	2.38	Quercetin-3-*O*-hexosyl-6″ acetate**,#	
46	15.18	$C_{20}H_{18}O_{11}$	433.0894	301.0338 [Y_0]$^-$ 271.0237 [Y_0-CHO]$^-$	353	0.01	Quercetin-3-*O*-arabinofuranoside (Avicularin)	Schieber, Conrad, Beifuss, & Carle, 2002; Schieber, Keller, Streker, Klaiber, & Carle, 2002
48	15.49	$C_{27}H_{28}O_{16}$	607.1286	505.0997 [M-H-$C_4H_8O_3$]$^-$ 463.0867 301.0332 [Y_0]$^-$	351	−3.13	Quercetin-3-[6″-(3-hydroxy-3-methylglutaryl)] β-hexoside**,#	
49	15.62	$C_{20}H_{18}O_{11}$	433.0769	301.0336 [Y_0]$^-$ 255.0316 [Y_0-CHO–OH]$^-$	255	−1.62	Quercetin-3-*O*-pentoside	Ramirez-Ambrosi et al., 2013
51	15.96	$C_{21}H_{20}O_{11}$	447.0931	301.0327 [Y_0]$^-$ 255.0284 [Y_0-CHO-OH]$^-$	254	1.94	Quercetin-3-*O*-rhamnoside (Quercitrin)	Ramirez-Ambrosi et al., 2013
52	16.22	$C_{22}H_{22}O_{12}$	477.1049	315.0488 [Y_0]$^-$ 300.0282 [Y_0-CH_3]$^-$ 271.0248 [Y_0-CH_3-CHO]$^-$	353	2.31	Isorhamnetin-3-*O*-galactoside**	Schieber, Conrad, Beifuss, & Carle, 2002; Schieber, Keller, Streker, Klaiber, & Carle, 2002
53	16.44	$C_{28}H_{32}O_{16}$	623.1612	315.0499 [Y_0]$^-$ 300.0267 [Y_0-CH_3]$^-$ 271.0240 [Y_0-CH_3-CHO]$^-$	354	−3.37	Isorhamnetin-3-*O*-rutinoside (Narcissin)**,#	Ramirez-Ambrosi et al., 2013

Table 1 (*continued*)

Peak	t_r	Molecular formula	$[M-H]^-$	[MS/MS]	PDA (nm)	Error (ppm)	Compound	References
Procyanidins								
18	7.60	$C_{60}H_{50}O_{24}$	576.1235*	865.1856 $[QM]^-$ 739.1675 $[HRF]^-$ 425.0858 $[RDA]^-$ 407.0754 $[RDA\text{-}H_2O]^-$ 577.1297 $[QM]^-$ 575.1172 $[QM]^-$	278	−5.21	Epicatechin tetramer (EC-4) (isomer II)	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
19	7.86	$C_{60}H_{50}O_{24}$	576.1243*	865.1856 $[QM]^-$ 739.1675 $[HRF]^-$ 425.0858 $[RDA]^-$ 407.0754 $[RDA\text{-}H_2O]^-$ 577.1297 $[QM]^-$ 575.1172 $[QM]^-$	279	−4.69	Epicatechin tetramer (EC-4) (isomer III)	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
20	8.83	$C_{75}H_{62}O_{30}$	720.1568*	1151.2404 $[QM]^-$ 865.1856 $[QM]^-$ 577.1320 $[QM]^-$ 575.1172 $[QM]^-$ 425.0842 $[RDA]^-$ 407.0783 $[RDA\text{-}H_2O]^-$ 287.0505 $[QM]^-$	278	2.20	Epicatechin pentamer (EC-5) (isomer I)	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
21	8.98	$C_{45}H_{38}O_{18}$	865.1968	739.1626 $[HRF]^-$ 577.1325 $[QM]^-$ 425.0858 $[RDA]^-$ 407.0728 $[RDA\text{-}H_2O]^-$ 287.0550 $[QM]^-$	281	−1.96	Epicatechin trimer (EC-3) (isomer III)	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
22	9.37	$C_{79}H_{78}O_{44}$	864.1890*	1153.2242 $[QM]^-$ 577.1206 $[QM]^-$ 575.1248 $[QM]^-$ 425.0844 $[RDA]^-$ 407.0707 $[RDA\text{-}H_2O]^-$ 287.0629 $[QM]^-$	276	3.74	Epicatechin hexamer (EC-6)	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
23	9.40	$C_{75}H_{62}O_{30}$	720.1571*	1151.2404 $[QM]^-$ 865.1856 $[QM]^-$ 577.1320 $[QM]^-$ 575.1172 $[QM]^-$ 425.0842 $[RDA]^-$ 407.0783 $[RDA\text{-}H_2O]^-$ 287.0505 $[QM]^-$	279	2.62	Epicatechin pentamer (EC-5) (isomer II)	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
24	9.41	$C_{105}H_{86}O_{42}$	1008.2226*	1153.2242 $[QM]^-$ 865.1958 $[QM]^-$ 739.1429 $[HRF]^-$ 575.1207 $[QM]^-$ 425.0864 $[RDA]^-$ 407.0972 $[RDA\text{-}H_2O]^-$	279	−1.37	Epicatechin heptamer (EC-7)**	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
25	9.51	$C_{30}H_{26}O_{12}$	577.1345	425.0869 $[RDA]^-$ 407.0754 $[RDA\text{-}H_2O]^-$ 289.0697 $[QM]^-$	279	−1.21	(Epi)catechin dimer	Gu et al., 2003
26	9.91	$C_{60}H_{50}O_{24}$	576.1240*	865.1856 $[QM]^-$ 739.1675 $[HRF]^-$ 425.0858 $[RDA]^-$ 407.0754 $[RDA\text{-}H_2O]^-$ 577.1297 $[QM]^-$ 575.1172 $[QM]^-$	278	3.22	Epicatechin tetramer (EC-4) (isomer IV)	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
27	10.29	$C_{45}H_{38}O_{18}$	865.1967	739.1626 $[HRF]^-$ 577.1325 $[QM]^-$ 425.0858 $[RDA]^-$ 407.0728 $[RDA\text{-}H_2O]^-$ 287.0550 $[QM]^-$	279	−2.54	Epicatechin trimer (EC-3) (isomer IV)	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
28	10.44	$C_{75}H_{62}O_{30}$	720.1573*	1151.2404 $[QM]^-$ 865.1856 $[QM]^-$ 577.1320 $[QM]^-$ 575.1172 $[QM]^-$ 425.0842 $[RDA]^-$ 407.0783 $[RDA\text{-}H_2O]^-$ 287.0505 $[QM]^-$	278	2.36	Epicatechin pentamer (EC-5) (isomer III)	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
29	10.99	$C_{75}H_{62}O_{30}$	720.1574*	1151.2404 $[QM]^-$ 865.1856 $[QM]^-$ 577.1320 $[QM]^-$ 575.1172 $[QM]^-$ 425.0842 $[RDA]^-$ 407.0783 $[RDA\text{-}H_2O]^-$ 287.0505 $[QM]^-$	278	2.64	Epicatechin pentamer (EC-5) (isomer IV)	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013

Table 1 (*continued*)

Peak	t_r	Molecular formula	[M-H]$^-$	[MS/MS]	PDA (nm)	Error (ppm)	Compound	References
Procyanidins								
33	12.28	$C_{79}H_{78}O_{44}$	864.1892*	1153.2242 [QM]$^-$ 577.1206 [QM]$^-$ 575.1248 [QM]$^-$ 425.0844 [RDA]$^-$ 407.0707 [RDA-H_2O]$^-$ 287.0629 [QM]$^-$	279	3.70	Epicatechin hexamer (EC-6) (isomer I)	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
37	13.14	$C_{30}H_{26}O_{12}$	577.1338	425.0869 [RDA]$^-$ 407.0754 [RDA-H_2O]$^-$ 289.0697 [QM]$^-$	279	−2.43	(Epi)catechin dimer	Gu et al., 2003
40	13.92	$C_{45}H_{38}O_{18}$	865.1955*	739.1626 [HRF]$^-$ 577.1325 [QM]$^-$ 425.0858 [RDA]$^-$ 407.0728 [RDA-H_2O]$^-$ 287.0550 [QM]$^-$	283	0.12	Epicatechin trimer (EC-3) (isomer V)	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013

* Detected as [M-2H]$^{2-}$.
** Reported for the first time in Annurca apple extract.
\# Not detected in Red Delicious extract.

Table 2
Oligomeric procyanidins detected by HILIC–ESI–IT-TOF.

DP	Molecular formula	HRMW	[M-H]$^-$	[M-2H]$^{2-}$	[MS/MS]	PDA (nm)	Error (ppm)	Compound	References
2	$C_{30}H_{26}O_{12}$	578.1336	577.1331	–	425.0869 [RDA]$^-$ 407.0754 [RDA-H_2O]$^-$ 289.0697 [QM]$^-$	279	−3.64	Epicatechin Dimer (procyanidin B2)	Gu et al., 2003
3	$C_{45}H_{38}O_{18}$	866.2058	865.1984	–	739.1626 [HRF]$^-$ 577.1325 [QM]$^-$ 425.0858 [RDA]$^-$ 407.0728 [RDA-H_2O]$^-$ 287.0550 [QM]$^-$	283	−0.12	Epicatechin trimer	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
4	$C_{60}H_{50}O_{24}$	1154.2692	1153.2652	–	865.1856 [QM]$^-$ 739.1675 [HRF]$^-$ 425.0858 [RDA]$^-$ 407.0754 [RDA-H_2O]$^-$ 577.1297 [QM]$^-$ 575.1172 [QM]$^-$	281	−1.47	Epicatechin tetramer	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
5	$C_{75}H_{62}O_{30}$	1442.3284	–	720.1569	1151.2404 [QM]$^-$ 865.1856 [QM]$^-$ 577.1320 [QM]$^-$ 575.1172 [QM]$^-$ 425.0842 [RDA]$^-$ 407.0783 [RDA-H_2O]$^-$ 287.0505 [QM]$^-$	280	−2.08	Epicatechin pentamer	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
6	$C_{90}H_{74}O_{36}$	1730.3960	–	864.1870	1153.2242 [QM]$^-$ 577.1206 [QM]$^-$ 575.1248 [QM]$^-$ 425.0844 [RDA]$^-$ 407.0707 [RDA-H_2O]$^-$ 287.0629 [QM]$^-$	279	−2.55	Epicatechin hexamer	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
7	$C_{105}H_{86}O_{42}$	2018.4594	–	1008.2226	1153.2242 [QM]$^-$ 865.1958 [QM]$^-$ 739.1429 [HRF]$^-$ 575.1207 [QM]$^-$ 425.0864 [RDA]$^-$ 407.0972 [RDA-H_2O]$^-$	280	−1.39	Epicatechin heptamer**	Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013
8	$C_{120}H_{98}O_{48}$	2306.5096	–	1152.2507	1008.7159 [HRF-H_2O]$^-$ 863.1777 [QM]$^-$ 737.1504 [HRF]$^-$ 575.1162 [QM]$^-$ 449.0860 [HRF] 425.0764 [RDA]$^-$ 407.0871 [RDA-H_2O]$^-$	279	−4.34	Epicatechin octamer**	Lin et al., 2014

** Oligomers described for the first time in Annurca apple extract

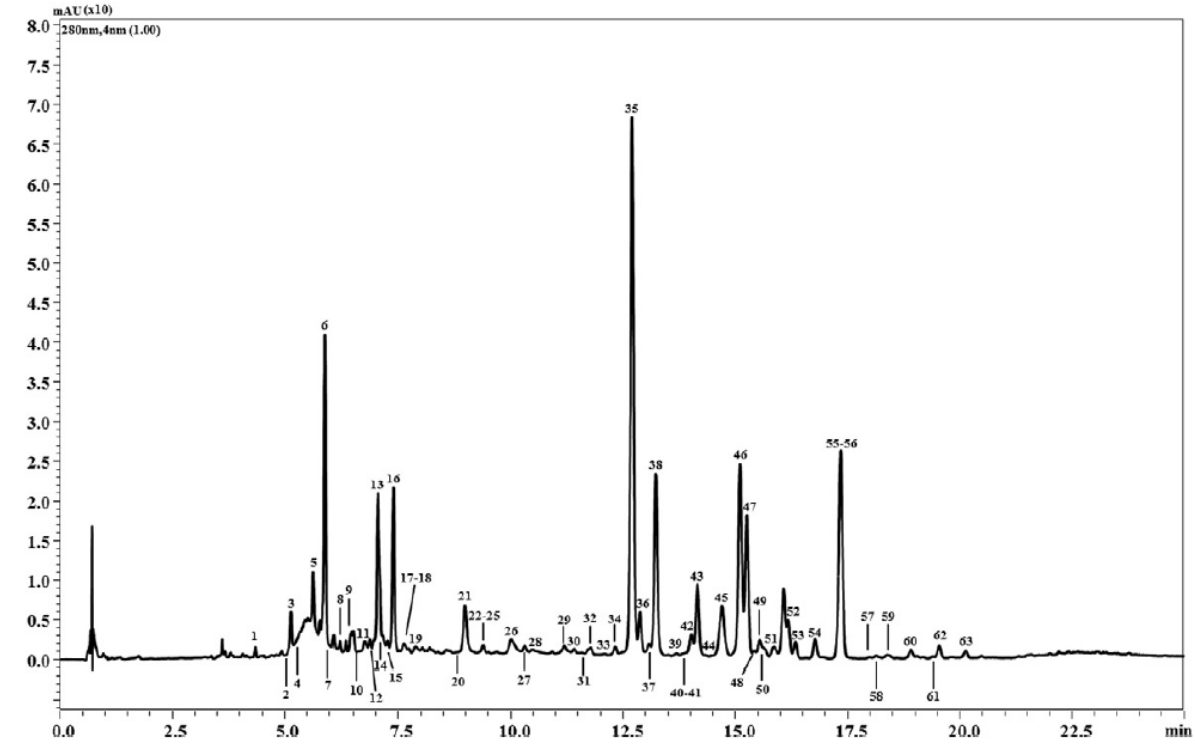

Fig. 1. RP-HPLC–UV chromatogram recorded at 280 nm of Annurca extract.

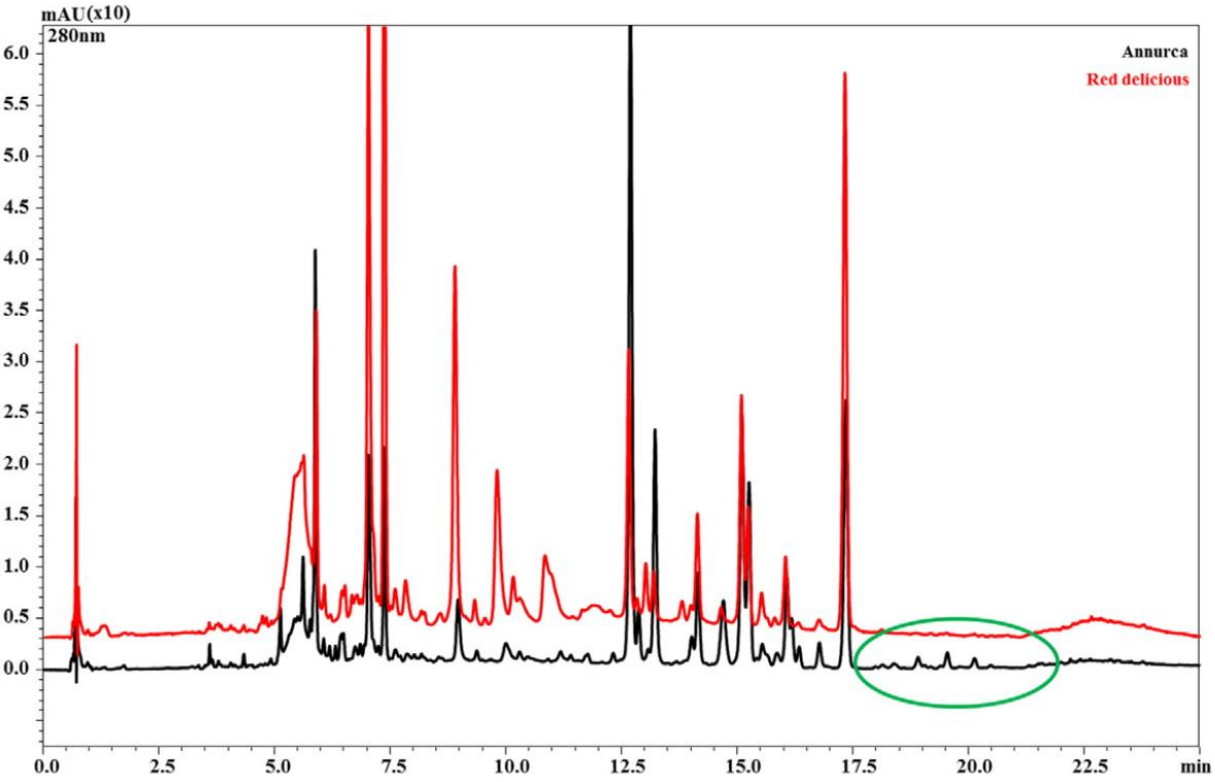

Fig. 2. RP-UHPLC–UV comparison between the profiles of Annurca and Red Delicious extract showing the absence of isorhamnetin derivatives.

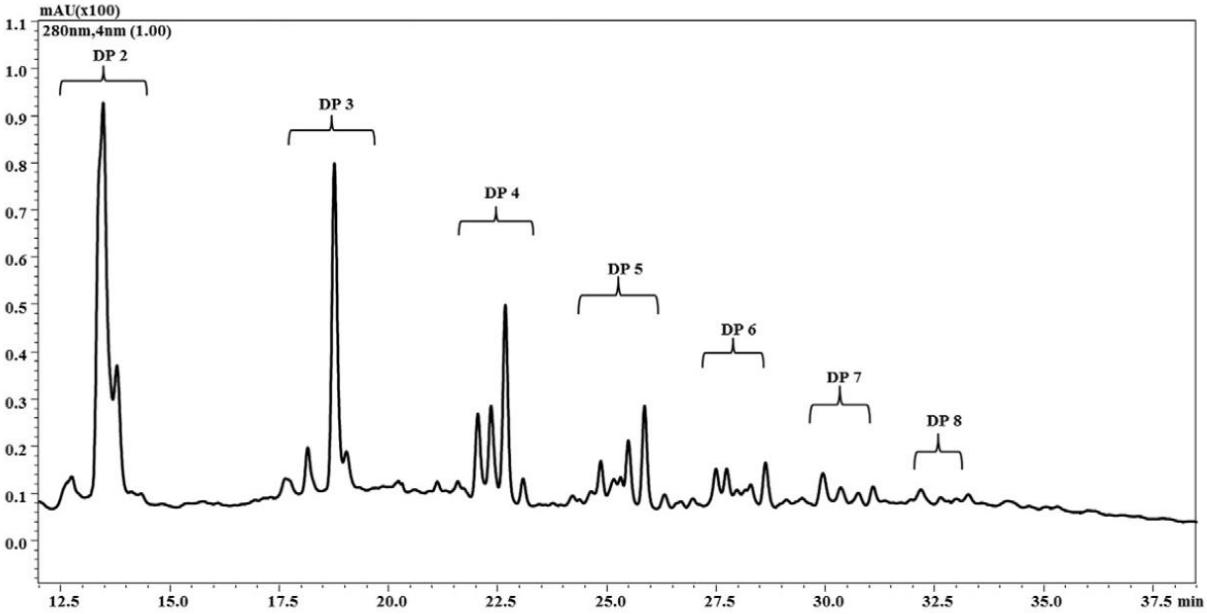

Fig. 3. Expansion of HILIC chromatogram recorded at 280 nm of Annurca extract showing the separation of procyanidin oligomers from DP 2 to 8.

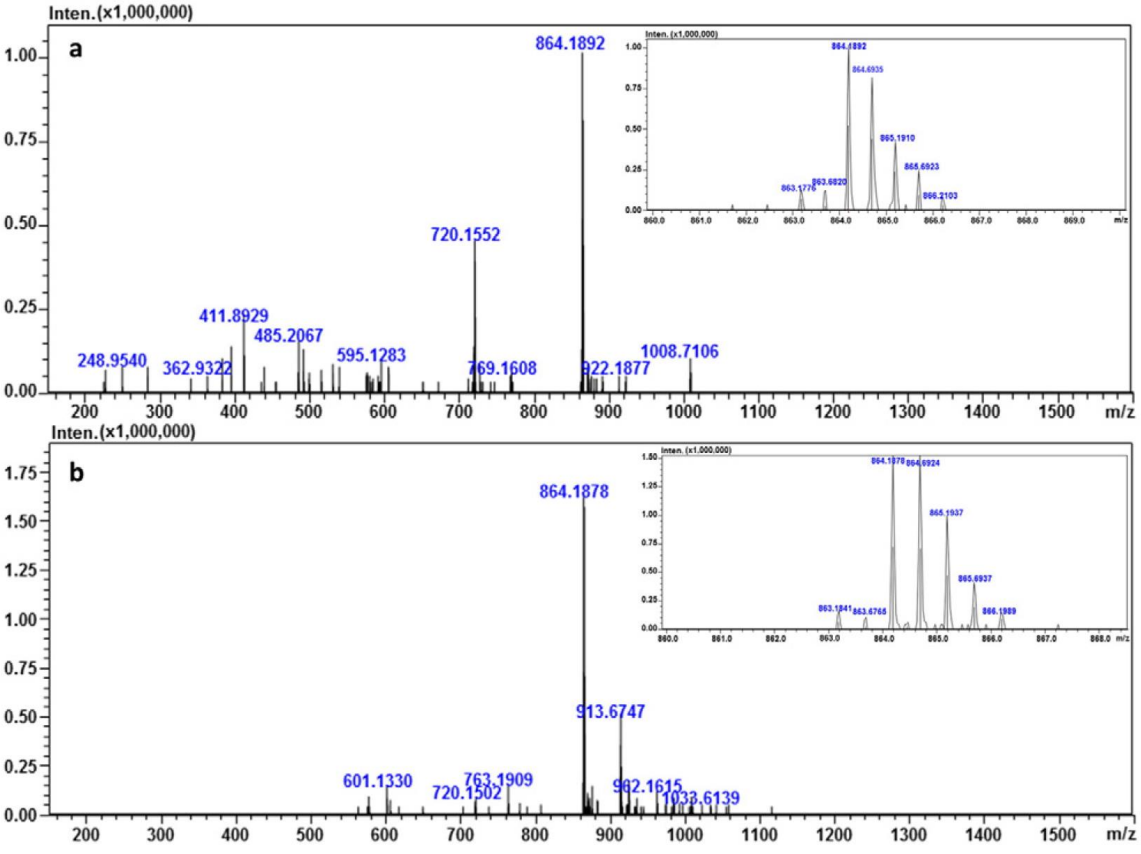

Fig. 4. a,b: Comparison of full scan MS intensities for RP-UHPLC (4a) and HILIC (4b) in the detection of epicatechin hexamer.