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HPLC-DAD-ESI-QTOF-MS and HPLC-FLD-MS as valuable tools for the determination of phenolic and other polar compounds in the edible part and by- products of avocado

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A B S T R A C T

Avocado is a tropical fruit increasingly cultivated around the world due to global interest and rising consumption. Thus, there is also a surge in avocado by-products that needs assessment. The aim of this work is to compare the phenolic profile of avocado pulp, peel and seed when the fruit is at optimal ripeness for consumption and when overripe. Two analytical techniques were used: (1) HPLC-DAD-ESI-QTOF-MS was used for the first time to determine phenolic and other polar compounds in avocado peel and seed. Phenolic compounds quantified with these methods were in higher concentration in overripe than in pulp and seed of optimally ripe fruit. (2) HPLC-FLD-MS was used to specifically determine flavan-3-ols. Procyanidins to degree of polymerization 13 have been quantified singularly here for the first time. In addition, A- and B-type procyanidins from the degree of polymerization 2 to 6 were differentiated and quantified. The procyanidin con-centration increased after ripening probably due to the release of tannins linked to cell-wall structures. Because of this situation and the presence of A-type procyanidins, avocado peel and seed from overripe fruit, the main by-products of avocado processing, hold interest for developing functional foods, nutraceuticals and cosmetics.

Keywords: Avocado, HPLC-DAD-ESI-QTOF-MS, Phenolic compounds, Flavan-3-ols, By-product

1. Introduction

Avocado, *Persea americana* Miller, of family Lauraceae, is a large drupe with the highest oil content of all fruits, except perhaps the olive. It is an evergreen tree with the feature that avocado fruits mature on the trees and ripen after harvest. Indigenous to Tropical America, today the avocado tree is cultivated in various subtropical countries (Borrone et al., 2009). Among the numerous varieties of avocado fruit around the world, the cultivar 'Hass' dominates the international market (Rodríguez-Carpena, Morcuende, Andrade, Kylli, & Estévez, 2011). Avocado production has considerably expanded in recent years due to rising consumption ("Food and Agriculture Organization of the United Nations Statistics Division" (FAOSTAT)).

Avocado is generally recognized as a popular and healthy food source supplying proteins and lipids to the human diet. The pulp contains several bioactive phytochemicals including carotenoids, B vitamins, vitamins C and E, D-mannoheptulose, β-sitosterol, and persenone A and B. These constituents have demonstrated *in vitro* antifungal, antitumor and antioxidant activities (Lu et al., 2005). Avocado pulp also has *in vitro* growth-inhibitory effects against cancer and is also well known for its beneficial skin properties. The unsaponifiable fraction of avocado pulp in combination with unsaponifiable soybean oil is used to treat osteoarthritis, and some research shows its anti-carcinogenic and anti-inflammatory effects (Boileau et al., 2009; Ding, Chin, Kinghorn, & D'Ambrosio, 2007; Lu et al., 2005). Although not as thoroughly studied as the pulp, avocado peel and seed reportedly contain great amounts of phenolic compounds and display a higher antioxidant activity than the pulp (Rodríguez-Carpena et al., 2011; Wang, Bostic, & Gu, 2010). The phenolic content and antioxidant capacity of avocado seeds and peels are several-fold greater than that reported for raw blueberry, which is known for its high antioxidant capacity (Ayala-Zavala et al., 2011).

Industrially, avocados are processed into oil and paste, which leaves 21–30% of the fruit weight to be discarded (Rodríguez-Carpena et al., 2011; Wang et al., 2010). These waste products represent a potential source of molecules with applications in the food, pharmaceutical, and cosmetic industries.

Thus, the aim of this work was to comprehensively determine the distribution of the phenolic compounds in extracts of seed, peel and pulp of the 'Hass' avocado cultivar by HPLC-DAD-ESI-QTOF-MS. Flavan-3-ols were also determined by HPLC-FLD-MS to quantify all polymerization grades of procyanidins. To the best of our knowledge, the phenolic compounds of peel and seed of avocado have never been analyzed before by HPLC-DAD-ESI-QTOF-MS.

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2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade acetic acid and HPLC-MS-grade acetonitrile were purchased from Fisher Scientific (Leicestershire, UK), and methanol from Panreac (Barcelona, Spain). Double-deionized water was obtained with a Milli-Q system from Millipore (Bedford, MA, USA). Ferulic acid, catechin, procyanidin B2, chlorogenic acid, tyrosol, rutin, gallic acid, hydroxybenzoic acid quinic acid and citric acid were acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Samples

About 10 kg of 'Hass' avocado from the subtropical coast of Granada were provided by Miguel García Sánchez e Hijos, S.A. (Motril, Spain) in July 2014. Half of the fruits were left in the laboratory until the optimal consumption ripeness (CRA), whereas half of avocados were left until overripeness (ORA). Then, pulp, peel and seed of the samples were manually separated in an ice bath, frozen at −80 °C and freeze-dried in a lyophilizer (Advantage Plus EL-85 freeze dryer, SP Scientific, Ipswich, Suffolk, UK). Afterwards, dried samples were milled (IKA M20-IKAWERKE GmbH & Co. KG, Staufen, Germany) and kept at −18 °C until used.

2.3. Extraction of phenolic compounds

A solid-liquid extraction was used to obtain the polar fraction of avocado pulp, peel and seed following a protocol from the literature with slight modifications (García-Salas, Gómez-Caravaca, Morales-Soto, Segura-Carretero, & Fernández-Gutiérrez, 2015). Briefly, 2 g of freeze-dried sample powder were extracted with 20 mL hexane to eliminate lipids and then dissolved in 15 mL of a solution of methanol/water (80:20, v/v). The mixture was placed in an ultrasonic bath for 15 min at room temperature and then centrifuged for 15 min at 1000 g. The supernatant was removed, and the extraction was repeated twice more. The supernatants were collected, evaporated, and reconstituted in 2 mL of methanol/water (80:20, v/v). The final extracts were filtered with regenerated cellulose filters 0.2 µm (Millipore, Bedford, MA, USA) and stored at −18 °C until analyzed.

2.4. Determination of phenolic and other polar compounds by HPLC-DAD-ESI-QTOF-MS

An Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, autosampler, binary pump, and DAD was used for the chromatographic determination.

A Poroshell 120 EC-C18 (4.6 mm × 100 mm, particle size 2.7 µm) (Agilent Technologies) was used to separate the compounds. Due to the different nature of the three matrices analyzed (especially avocado pulp, which has a high number of phenolic acid derivatives compared to avocado peel and seed, which contain mainly procyanidins, some flavonoids and a lower quantity of phenolic acid derivatives), two analytical methods were used. Avocado pulp was analyzed using water containing 1% acetic acid as the solvent system A and acetonitrile as solvent system B and the following gradient elution: 0 min, 1% B; 15 min, 40% B; 16 min, 40% B; 19 min, 100% B; 21 min, 1% B; and 23 min, 1% B. The sample volume injected was 3 µL and the flow rate used was 1.6 mL min^{-1}. Avocado peel and seed were analyzed using the same gradient elution. Mobile phase A was water containing 1% acetic acid and mobile phase B was acetonitrile and they were used as follows: 0 min, 5% B; 4 min, 15% B; 5 min, 16% B; 8 min, 18% B; 12 min, 100% B; 14 min, 100% B; 16 min, 5% B; and 18 min, 5% B. The sample volume injected was 3 µL and the flow rate used was 1.2 mL min^{-1}. The column was maintained at 25 °C. All samples were analyzed in triplicate.

UV spectra were recorded from 200 to 600 nm, whereas the chromatograms were registered at 240, 280, and 330 nm. The effluent from the HPLC column was split using a T-type phase separator before introducing it into the mass spectrometer (split ratio 1:3). MS analyses were carried out using a 6540 Agilent Ultra-High-Definition Accurate-Mass Q-TOF-MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization interface in negative ionization mode under the following conditions: drying gas flow (N_2), 12.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 370 °C; capillary voltage, 3500 V; fragmentor voltage and scan range, 3500 V and *m/z* 50–1500, respectively. Automatic MS/MS experiments were conducted using the following collision-energy values: *m/z* 100, 30 eV; *m/z* 500, 35 eV; *m/z* 1000, 40 eV; and *m/z* 1500, 45 eV. Reference mass correction of each sample was performed with a continuous infusion of Agilent TOF mixture containing two mass references. The detection window was set to 100 ppm. Data acquisition (2.5 Hz) in the centroid mode was governed via the Agilent MassHunter Workstation. Integration and data elaboration were carried out using MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA).

The calibration curves using ferulic acid, chlorogenic acid, tyrosol, rutin, gallic acid, hydroxybenzoic acid, quinic acid and citric acid were prepared for the quantification of phenolic compounds and organic acids. Quantification was performed using DAD chromatograms of ferulic acid, tyrosol, rutin, gallic acid, hydroxybenzoic acid at $\lambda = 280$ nm and chlorogenic acid at $\lambda = 330$ nm. The calibration curves of citric acid and quinic acid were performed using the MS chromatogram. The calibration curves were prepared from the limit of quantification (LOQ) to 125 µg/mL. All calibration curves showed good linearity among different concentrations ($r > 0.999$). The analytical parameters of the method such as limit of detection (LOD), LOQ, intraday and interday precision (expressed as % RSDs) of the retention times and the total peak area are summarized in Table 1.

The calibration curve of citric acid was used to quantify citric and succinic acid; the calibration curve of ferulic acid was used to quantify hydroxycinnamic acid derivatives; the calibration curve of quinic acid was used to quantify quinic acid, the calibration curve of chlorogenic acid was used to quantify quinic acid derivatives; the calibration curve of tyrosol was used to quantify hydroxytyrosol glucoside, tyrosol glucoside and tyrosol hexoside-pentoside; the calibration curve of rutin was used to quantify rutin and other quercetin derivatives; the calibration curve of gallic acid was used to quantify octyl gallate; and the calibration curve of hydroxybenzoic acid was used to quantify protocatechuic acid-4-glucoside and vanillic acid glucoside. It has to be taken into account that the response of the standards can differ from the response of the derivatives present in avocado samples, and consequently the quantification of these compounds is only an estimation of their actual concentrations.

2.5. Determination of flavan-3-ols by HPLC-FLD-MS

An Agilent 1200 Series (Agilent Technologies, Palo Alto, CA, USA), equipped with a binary pump delivery system, a degasser, an autosampler, a fluorimetric detector (FLD) and a mass-spectrometer

Table 1
Precision, limit of detection and limit of quantification of the methods proposed.

Analyte	Pulp						Peel and seed					
	Intraday RSD (%) Rt	Interday RSD (%) Rt	Intraday RSD (%) Area	Interday RSD (%) Area	LOD (μg/mL)	LOQ (μg/mL)	Intraday RSD (%) Rt	Interday RSD (%) Rt	Intraday RSD (%) Area	Interday RSD (%) Area	LOD (μg/mL)	LOQ (μg/mL)
Citric acid	0.57	1.54	0.66	1.27	0.130	0.768	0.62	1.38	0.67	1.31	0.170	0.683
Gallic acid	0.13	1.31	0.56	1.07	0.037	0.128	0.11	1.02	0.52	0.98	0.096	0.291
Quinic acid	0.78	1.04	0.31	0.83	0.097	0.301	0.65	0.96	0.28	0.69	0.102	0.316
Chlorogenic acid	2.63	2.97	0.42	0.94	0.045	0.194	2.34	2.85	0.45	0.87	0.035	0.112
Tyrosol	0.85	1.83	0.59	1.14	0.064	0.232	0.74	1.43	0.49	1.04	0.051	0.166
Ferulic acid	0.84	1.74	0.43	1.02	0.080	0.255	0.71	1.44	0.40	0.79	0.083	0.263
Rutin	1.67	2.31	0.52	0.99	0.015	0.046	1.39	2.17	0.37	0.84	0.012	0.041
Hydroxybenzoic acid	0.53	1.36	0.38	0.88	0.019	0.067	0.77	1.21	0.35	0.91	0.049	0.157

detector (MSD) was used for the analyses. The methodology was the same reported by Verardo et al. (2015).

Avocado samples were extracted using a solution of acetone/water (4:1, v/v). A Develosil Diol 100 Å column 5 μm, 250 × 4.6 mm ID (Phenomenex, Torrance, CA, USA) was used. Mobile phase A and B consisted of an acidic acetonitrile ((A), CH_3CN:CH_3COOH, 98:2; v/v) and acidic aqueous methanol ((B),CH_3OH:H_2O: CH_3COOH, 95:3:2; v/v/v). The gradient elution was the following: 7% B for 3 min, 37.6% B for 57 min, and 100% B for 7 min. Then the initial conditions were set, 7% B during 5 min. Fluorescence detection was performed with an excitation wavelength of 230 nm and an emission wavelength of 321 nm. The injection volume was 5 μL. All the analyses were carried out at 35 °C.

Mass spectrometer analyses were carried out using an electrospray ionization (ESI) interface under the following conditions: drying gas flow (N_2), 9.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 350 °C; capillary voltage, 3500 V; fragmentor voltage and scan range variables. The fragmentor and *m/z* ranges used for HPLC-ESI/MS analyses were: 120 V and *m/z* 50–1000, 140 V and *m/z* 1000–2000.

2.6. Statistical analysis

One-way analysis of variance, ANOVA (Tukey's honest significant difference multiple comparison), was evaluated using Statistica 8.0 software (StatSoft, Tulsa, OK, USA), and p values < 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Identification of phenolic and other polar compounds by HPLC-DAD-ESI-QTOF-MS

Avocado pulp, peel and seed extracts obtained by solid-liquid extraction were analyzed using an HPLC coupled to DAD and ESI-QTOF-MS in the negative ionization mode in order to identify phenolic and other polar compounds. Peak identification was performed on basis of their relative retention time values, their UV–Vis spectra and mass spectra obtained using ESI-QTOF-MS together with information previously reported in the literature. Table 2 summarizes the tentatively identified compounds in the three fractions of avocado. Compounds which were not identified in pulp were subsequently numbered in peel and seed.

Flavan-3-ols have not been included in this identification table because specific analyses for these compounds have been performed by HPLC-FLD-MS.

3.1.1. Avocado pulp

A total of 23 compounds were tentatively identified in avocado pulp (Table 2). These belonged to various metabolite types that included organic acids, amino acids, sugars, nucleosides, phenolic acids (hydroxybenzoic and hydroxycinnamic), phenolic alcohols and iridoids.

A sugar (perseitol), three organic acids (quinic acid, citric acid and succinic acid), and an amino acid (uridine) were identified, in agreement with previous studies on avocado pulp (Contreras-Gutiérrez et al., 2013; Hurtado-Fernández, Carrasco-Pancorbo, & Fernández-Gutiérrez, 2011; Hurtado-Fernández, Pacchiarotta, et al., 2011).

In addition, seven hydroxycinnamic acid derivatives (sinapic acid-C-hexoside, *p*-coumaric acid glucoside and one of its isomers, ferulic acid glucoside and its isomer, *p*-coumaric acid rutinoside and coumaric acid) and one hydroxybenzoic acid derivative (octyl gallate) were also identified, as previously described (Hurtado-Fernández, Pacchiarotta, et al., 2011; Hurtado-Fernández, Carrasco-Pancorbo et al., 2011).

Furthermore, another six hydroxycinnamic acid derivatives that, to our knowledge, had not been identified in avocado pulp before were found using the methodology previously described in section 2.4 by HPLC-DAD-ESI-QTOF-MS. Protocatechuic acid linked to a hexose (peak 6) was identified at *m/z* 315.0722 and a retention time 3.17 min in avocado pulp. The fragment at *m/z* 153 corresponded to a protocatechuic acid moiety. This compound could be tentatively identified as protocatechuic acid-4- glucoside, as previously described in literature for other fruits and plants (Gómez-Caravaca, Segura-Carretero, Fernández-Gutiérrez, & Caboni, 2011; Schuster & Herrmann, 1985). Peak 9, *m/z* 341.0886 at 4.71 min, presented fragments at *m/z* 161 and 179. This finding could indicate the loss of a hexoside moiety (probably glucoside) and the presence of caffeic acid. Thus, this compound was identified as caffeic acid glucoside (Verardo, Gomez-Caravaca, Segura-Carretero, Caboni, & Fernández-Gutiérrez, 2011). Peak 13 was identified as *p*-coumaric acid hexoside pentoside. Its fragmentation pattern showed fragments at *m/z* 323 due to the loss of a pentoside moiety, at *m/z* 307 because of the loss of a hexoside moiety. Characteristic fragments of coumaric acid moieties were also present (*m/z* 163 and 145).

Three isomers of feruloylquinic acid (peaks 19, 21 and 22) were found at *m/z* 367.103 and retention times of 7.18, 7.58 and 7.95 min, respectively. They were identified as 3, 5 and 4-feruloylquinic acids, respectively based on their fragmentation pattern and the elution order found in literature for similar separation conditions (Clifford, Johnston, Knight, & Kuhnert, 2003). Finally, at *m/z* 469.1354 and a retention time of 8.20 min, agoniadin was identified. Fragments at

Table 2
Identification of phenolic and other polar compounds in pulp, peel and seed of avocado by HPLC-DAD-qTOF-MS.

Peak	Proposed compound	Retention time (min)	UV λ_{max} (nm)	*m/z* experimental $[M-H]^-$	*m/z* calculated $[M-H]^-$	Fragments MS/MS	Molecular formula	Error (ppm)	Score
Pulp									
1	perseitol	0.58	242/265	211.0829	211.0823	101	$C_7H_{16}O_7$	2.7	97.51
2	quinic acid[a]	0.74	230/262	191.0571	191.0561	111	$C_7H_{12}O_6$	5.7	93.18
3	citric acid[a]	1.16	230	191.0200	191.0197	111, 101, 113	$C_6H_8O_7$	1.4	99.04
4	Succinic acid	1.58	230/265	117.0194	117.0193	100	$C_4H_6O_4$	0.93	86.35
5	uridine	1.59	228/262	243.0625	243.0631	227	$C_9H_{12}N_2O_6$	3.48	96.54
6	protocatechuic acid-4-glucoside	3.17	240/311	315.0722	315.0722	108, 153	$C_{13}H_{16}O_9$	0.08	99.63
7	penstemide	3.81	230/272	443.1934	443.1923	101,113	$C_{21}H_{32}O_{10}$	1.72	98.11
8	sinapic acid-C-hexoside	4.50	234/306	385.1143	385.114	223	$C_{17}H_{22}O_{10}$	0.64	99.37
9	caffeic acid glucoside	4.71	237/308	341.0886	341.0878	161, 133, 179	$C_{15}H_{18}O_9$	2.36	96.88
10	penstemide isomer I	5.12	230/272	443.1929	443.1923	101, 113, 161	$C_{21}H_{32}O_{10}$	1.13	98.82
11	tyrosol-hexoside-pentoside	5.19	228/278	431.1562	431.1559	299,119, 113, 137, 131, 149,179, 161	$C_{19}H_{28}O_{11}$	0.85	98.51
12	*p*-coumaric acid glucoside	5.68	233/283/314	325.0935	325.0929	163,145	$C_{15}H_{18}O_8$	1.88	97.47
13	*p*-coumaric acid hexoside pentoside	5.96	284/315	457.1360	457.1351	145,163,307,323	$C_{20}H_{26}O_{12}$	1.75	98.27
14	*p*-coumaric acid glucoside isomer I	6.05	233/283/314	325.0934	325.0929	145, 117, 119	$C_{15}H_{18}O_8$	1.52	98.59
15	ferulic acid glucoside	6.22	240/295/329	355.1039	355.1035	193, 175	$C_{16}H_{20}O_9$	1.3	97.83
16	*p*-coumaric acid rutinoside	6.30	240/279/315	471.1516	471.1508	163, 145	$C_{21}H_{28}O_{12}$	1.72	97.92
17	ferulic acid glucoside isomer I	6.54	240/295/329	355.1039	355.1035	193, 175	$C_{16}H_{20}O_9$	1.17	99.25
18	octyl gallate	6.55	272/322	281.1393	281.1394	121, 139	$C_{15}H_{22}O_5$	0.57	98.83
19	3-feruloylquinic acid	7.18	282/315	367.1035	367.1035	193, 191	$C_{17}H_{20}O_9$	0.01	98.97
20	coumaric acid	7.49	236/279/308	163.0396	163.0401	119	$C_9H_8O_3$	2.26	98.64
21	5-feruloylquinic acid	7.58	235/288/316	367.1039	367.1035	173, 191	$C_{17}H_{20}O_9$	0.97	97.56
22	4-feruloylquinic acid	7.95	281/312	367.1039	367.1035	173, 191	$C_{17}H_{20}O_9$	1.06	99.42
23	agoniadin	8.20	240/290/317	469.1354	469.1351	163, 145	$C_{21}H_{26}O_{12}$	0.52	98.26
Peel									
1	perseitol	0.868	242/265	211.0825	211.0823	101	$C_7H_{16}O_7$	1.0	99.77
2	quinic acid	0.914	230/262	191.0566	191.0561	111	$C_7H_{12}O_6$	2.38	98.29
10	penstemide	4.344	230/272	443.1934	443.1923	101, 113	$C_{21}H_{32}O_{10}$	2.4	97.01
24	chlorogenic acid[a]	4.496	234/295/326	353.0889	353.0878	191	$C_{16}H_{18}O_9$	2.92	96.31
25	quercetin-diglucoside	6.456	238/280/352	625.1418	625.141	301	$C_{27}H_{30}O_{17}$	1.02	98.86
26	quercetin-3-O-arabinosyl-glucoside	7.059	236/279/354	595.1308	595.1305	301	$C_{26}H_{28}O_{16}$	0.36	99.42
27	rutin[a]	9.855	234/282/350	609.1474	609.1461	301	$C_{27}H_{30}O_{16}$	1.98	97.34
Seed									
1	perseitol	0.872	242/265	211.0827	211.0823	101	$C_7H_{16}O_7$	1.88	99.01
2	quinic acid	0.917	230/262	191.0567	191.0561	111	$C_7H_{12}O_6$	4.05	93.95
3	citric acid[a]	1.183	230	191.0197	191.0197	111, 101, 113	$C_6H_8O_7$	0.04	99.88
28	hydroxytyrosol glucoside	3.126	234/280	315.1094	315.1085	135, 153	$C_{14}H_{20}O_8$	2.48	96.57
29	1-caffeoylquinic acid	3.585	239/293/324	353.088	353.0878	191, 179, 135	$C_{16}H_{18}O_9$	0.55	99.84
30	tyrosol glucoside	4.012	229/276	299.1138	299.1136	119, 137	$C_{14}H_{20}O_7$	0.43	99.42
10	penstemide	4.344	234/295/326	443.1924	443.1923	101,113	$C_{21}H_{32}O_{10}$	0.18	99.74
31	3-O-*p*-coumaroylquinic acid	4.538	238/285/311	337.093	337.0929	163	$C_{16}H_{18}O_8$	0.27	99.83
32	4-caffeoylquinic acid	5.075	241/284/326	353.0887	353.0878	135, 173, 191	$C_{16}H_{18}O_9$	2.54	97.08
33	vanillic acid glucoside	6.366	239/279	329.0878	330.0951	123, 167	$C_{14}H_{18}O_9$	0.14	99.06
34	(1′S, 6′R)-8'-hydroxyabscisic acid beta-D-glucoside	8.546	242/274	441.177	441.1766	330,139	$C_{21}H_{30}O_{10}$	0.67	99.24

[a] Compounds checked against an authentic standard.

m/z 163 and 145 corresponding to the coumaric acid moiety of this compound were generated after the MS/MS analysis.

One phenolic alcohol was found at 5.19 min and *m/z* 431.1562. It generated different fragments, at *m/z* 137 that corresponded to a tyrosol moiety, at *m/z* 299 that matched the loss of a pentose moiety tyrosol hexoside and further fragmentation of *m/z* 299 resulted in a pattern matching that of tyrosol hexoside (Eyles et al., 2007). Therefore, this compound was identified as tyrosol hexoside pentoside. Moreover, two iridoids (peaks 7 and 10) were found at *m/z* 443.193 and presented fragments at *m/z* 101. Thus, these compounds were identified as penstemide and its isomer as previously described by other authors (Rodríguez-Pérez et al., 2013). These compounds have also been identified for the first time in avocado in this study.

3.1.2. Avocado peel

Seven compounds were identified in this fraction of avocado. Three of them, belonging to the flavonol family (peaks 25, 26 and 27), were quercetin derivatives: quercetin-diglucoside, quercetin-3-O-arabinosyl-glucoside and rutin. All showed a fragment at *m/z* 301 due to a quercetin moiety (Kosińska et al., 2012).

A derivative of caffeic acid such as chlorogenic acid was identified in avocado peel. It showed *m/z* 353.0889 and a fragment at *m/z* 191 due to the quinic acid moiety. This compound was also confirmed by the comparison with its commercial standard.

Perseitol, quinic acid and penstemide were also found in avocado peel. To the best of our knowledge, this is the first time that these compounds have been identified in avocado peel.

3.1.3. Avocado seed

A total of 11 compounds were identified in avocado seed. Perseitol, quinic acid, citric acid and penstemide were also found in the seed and they presented the same fragmentation pattern as in avocado peel. These compounds have not previously been described in avocado seed.

Two phenolic alcohol derivatives were identified in seed. Hydroxytyrosol 1-glucoside (peak 28) showed an *m/z* at 315.1094 and two fragments, one at *m/z* 153 corresponding to the presence of hydroxytyrosol and another one at *m/z* 135 due to the loss of a glucose moiety. Tyrosol hexoside was also found in seed. The presence of tyrosol derivatives in avocado seed has previously been described by other authors (Dabas, Shegog, Ziegler, & Lambert, 2013; Ramos-Jerz, Villanueva, Jerz, Winterhalter, & Deters, 2013).

(1′S, 6′R)-8′-hydroxyabscisic acid beta-D-glucoside was also identified in avocado seed (Ramos-Jerz et al., 2013).

Finally, four hydroxycinnamic acid derivatives were also found in avocado seed: 3-O-*p*-coumaroylquinic acid was previously described by other authors (Kosińska et al., 2012). Meanwhile, vanillic acid glucoside (peak 33) at *m/z* 329.0878 and fragments at *m/z* 123 and 167 indicating the presence of a vanillic acid moiety, was identified for the first time in avocado seed.

Two caffeoylquinic acid isomers were found in seed. The presence of the isomer corresponding to chlorogenic acid was ruled out because they did not match with the commercial standard of this compound. According to Clifford et al. (Clifford, Knight, & Kuhnert, 2005), peak 29 was identified as 1-caffeoylquinic acid because it showed a strong fragment at *m/z* 191 and a weak fragment at *m/z* 179, and peak 32 was identified as 4-caffeoylquinic acid because it was the only isomer that showed a MS/MS base peak at *m/z* 173. These specific chlorogenic derivative compounds were identified for the first time in avocado seed.

3.2. Quantification of phenolic compounds and other compounds

The pulp, peel and seed of CRA and ORA samples were quantified for phenolic compounds and organic acids. Each compound and also the total compound contents were compared between CRA and ORA. The phenolic content of fruits is known to be influenced by the degree of ripeness. However, few studies investigated phenolic and other polar metabolites in avocado (Hurtado-Fernández, Pacchiarotta, et al., 2011; Villa-Rodríguez, Molina-Corral, Ayala-Zavala, Olivas, & González-Aguilar, 2011). Only avocado pulp has been studied in this sense, and to our knowledge, there are no studies about the evolution of these compounds during fruit ripening in avocado by-products such as peel and seed.

Regarding avocado pulp (Table 3), quinic acid showed no significant differences between ORA and CRA pulp, whereas succinic acid proved to be significantly higher in ORA pulp than in CRA pulp. This agrees with previously reported results (Hurtado-Fernández, Pacchiarotta, et al., 2011). Citric acid was also significantly higher in ORA pulp than in CRA pulp. Most phenolic compound derivatives of avocado pulp were significantly higher in ORA than in CRA pulp. Sinapic acid-C-hexoside did not change with ripening, whereas only caffeic acid glucoside, tyrosol-hexoside-pentoside, *p*-coumaric acid glucoside, ferulic acid glucoside isomer I, coumaric acid and 4-feruloylquinic acid were less concentrated in ORA than in CRA pulp. Furthermore, the total content of these compounds in avocado pulp was 45.03 mg/100 g dry matter in ORA pulp and 38.79 mg/100 g in CRA pulp. A similar trend in avocado pulp has been detected by other authors (Hurtado-Fernández, Pacchiarotta, et al., 2011; Villa-Rodríguez et al., 2011). This is probably due to the effect of phenylalanine ammonia lyase (PAL), which has been demonstrated to increase in activity with the ripening of avocado (Martinez & Whitaker, 1995).

On the contrary, phenolic and other polar compounds in avocado peel were much lower in ORA than in CRA peel, 296.65 and 515.52 mg/100 g, respectively (Table 4). However, quercetin-3-O-arabinosyl-glucoside and rutin were significantly higher in ORA than in CRA peel. No qualitative differences were found between the two samples. It is possible that external conditions affect mainly the peel deteriorating these kinds of compounds.

Table 3
Phenolic and other polar compounds in avocado pulp expressed as mg/100 g dry matter[a].

Pulp compounds	CRA		ORA	
	Mean	SD	Mean	SD
quinic acid	0.12a	0.01	0.10a	0.00
citric acid	3.61b	0.19	3.89a	0.18
succinic acid	1.10b	0.09	1.96a	0.02
protocatechuic acid-4-glucoside	0.24a	0.01	0.27a	0.03
sinapic acid-C-hexoside	0.74a	0.002	0.74a	0.04
caffeic acid glucoside	0.98a	0.03	0.78b	0.00
tyrosol-hexoside-pentoside	2.28a	0.10	2.04b	0.06
p-coumaric acid glucoside	8.44b	0.15	9.35a	0.07
p-coumaric acid hexoside pentoside	1.06b	0.02	1.57a	0.00
p-coumaric acid glucoside isomer I	1.03a	0.02	0.56b	0.04
ferulic acid glucoside	1.95b	0.07	2.44a	0.10
p-coumaric acid rutinoside	1.64b	0.02	3.58a	0.08
ferulic acid glucoside isomer I	0.74a	0.02	0.66b	0.01
octyl gallate	0.95b	0.02	2.18a	0.17
3-feruloylquinic acid	0.77b	0.03	1.23a	0.09
coumaric acid	4.69a	0.05	2.76b	0.02
5-feruloylquinic acid	7.62b	0.09	11.55a	0.60
4-feruloylquinic acid	0.81a	0.03	0.67b	0.09
Total	38.75b	0.86	44.99a	1.01

CRA: optimal consumption ripening avocado; ORA over-ripped avocado.
Citric and succinic acid were quantified in mg equivalent of citric acid/100 g dry matter.
Quinic acid was quantified in mg equivalent of quinic acid/100 g dry matter.
Caffeic acid glucoside were quantified in mg equivalent of chlorogenic acid/100 g dry matter.
Sinapic acid-C-hexoside, p-coumaric acid glucoside, p-coumaric acid hexoside pentoside, p-coumaric acid glucoside isomer I, ferulic acid glucoside, p-coumaric acid rutinoside, ferulic acid glucoside isomer I and 3-feruloylquinic acid, coumaric acid, 5-feruloylquinic acid and 4-feruloylquinic acid were quantified in mg equivalent of ferulic acid/100 g dry matter.
Tyrosol-hexoside-pentoside was quantified in mg equivalent of tyrosol/100 g dry matter.
Octyl gallate was quantified in mg equivalent of gallic acid/100 g dry matter.
Protocatechuic acid-4-glucoside was quantified in mg equivalent of hydroxybenzoic acid/100 g dry matter.

[a] Different letters in the same row indicate significant differences ($p < 0.05$).

Table 4
Phenolic and other polar compounds in avocado peel expressed as mg/100 g dry matter[a].

Peel compounds	CRA		ORA	
	Mean	SD	Mean	SD
quinic acid	0.43a	0.01	0.12b	0.02
chlorogenic acid	189.89a	1.07	159.24b	0.82
quercetin-3,4'-diglucoside	270.05a	2.19	29.50b	3.73
quercetin-3-O-arabinosyl-glucoside	19.76b	0.22	60.78a	0.30
rutin	35.31b	0.61	46.96a	2.09
total	515.45a	0.00	296.61b	2.22

CRA: optimal consumption ripening avocado; ORA over-ripped avocado.
Quinic acid was quantified in mg equivalent of quinic acid/100 g dry matter.
Chlorogenic acid was quantified in mg equivalent of chlorogenic acid/100 g dry matter.
Quercetin-3,4′-diglucoside, quercetin-3-O-arabinosyl-glucoside, rutin were quantified in mg equivalent of rutin/100 g dry matter.

[a] Different letters in the same row indicate significant differences ($p < 0.05$).

Finally, avocado seed (Table 5) showed a significantly higher content of all single compounds in ORA seed than in CRA seed, except for hydroxytyrosol 1-glucoside, which decreases with ripening. Quinic acid content did not change with ripening. The total content of phenolic and other polar compounds in avocado seed was also higher in ORA seed (676.45 mg/100 g) than in CRA seed (400.07 mg/100 g).

3.3. Identification of flavan-3-ols by HPLC-FLD-MS

To identify flavan-3-ols, fluorimetric detection and mass spectrometry were used to confirm the peak identity. As previously described in literature, flavan-3-ols eluted according to their degree of polymerization (DP), firstly eluting the monomers and then the different oligomers (Robbins et al., 2009). A-type and B-type procyanidins (PCs) were identified in avocado peel and seed until the hexamer DP. This is the first time that avocado procyanidins have been differentiated in A-type and B-type from DP from 3 to 6. As reported by other authors, A-type procyanidins eluted before B-type procyanidins (Gu et al., 2002; Wallace & Giusti, 2010). Fig. 1 lists the flavan-3-ols identified in avocado pulp, peel and seed.

For avocado pulp, the first peak corresponded to the monomers catechin and epicatechin, and showed a molecular ion at *m/z* 289 and fragments ion at *m/z* 245 ($[M-H-CO_2]^-$) and *m/z* 139 (Retro Diels Alder fragmentation) (Verardo et al., 2015). B-type to DP 4 oligomeric procyanidins were identified in avocado pulp. A B-type PC dimer was found at *m/z* 577 and showed two principal fragments at *m/z* 425 and 289 (Pérez-Jiménez & Torres, 2012). The B-type PC trimer registered a molecular ion at *m/z* 865 and several fragments at *m/z* 739, 713 and 289. A B-type PC tetramer at *m/z* 1153 and fragments at *m/z* 865 and 577 was also found (Pérez-Jiménez & Torres, 2012; Verardo et al., 2015). Finally the polymers were detected.

Avocado seed and peel flavan-3-ols profiles were qualitatively similar. The monomer catechin/epicatechin was found in these fractions of avocado. PCs A and B-type from DP 2 to 13 were identified. Major PCs were identified by MS and FLD (DP from 2 to 5), whereas for PCs from DP up to 6, only FLD identification was possible. This was due to the limited ionization of big polymers and their lower sensibility in MS compared to FLD. However, identification was possible because peak pattern in all cases was the same.

The B-type PC dimer, trimer and tetramer had the same mass spectra as previously described for avocado pulp. A B-type PC pentamer was found at *m/z* 1441 and showed fragments at *m/z* 865 and 577.

Concerning A-type PCs, the A-type PC dimer showed a molecular ion at *m/z* 575 and a fragment at *m/z* 289; the A-type PC trimer presented a molecular ion at *m/z* 863 and fragments at *m/z* 577 and 289; the A-type PC tetramer had a molecular ion at *m/z* 1151 and fragments at *m/z* 577 and 289 and the A-type PC pentamer showed a molecular ion at *m/z* 1439 and fragments at *m/z* 577 and 289.

Table 5
Phenolic and other polar compounds in avocado seed expressed as mg/100 g dry matter[a].

Seed compounds	CRA		ORA	
	Mean	SD	Mean	SD
quinic acid	0.08a	0.01	0.08a	0.01
citric acid	4.63b	0.14	12.39a	0.30
hydroxytyrosol glucoside	38.95a	0.61	25.22b	1.32
1-caffeoylquinic acid	112.29b	0.41	243.78a	9.52
tyrosol glucoside	223.66b	1.33	339.14a	11.85
3-O-*p*-coumaroylquinic acid	7.01b	0.05	37.56a	1.73
4-caffeoylquinic acid	6.69b	0.05	10.39a	0.62
vanillic acid glucoside	6.74b	0.27	7.86a	0.01
total	400.05b	0.00	676.43a	24.74

CRA: optimal consumption ripening avocado; ORA over-ripped avocado.
Citric acid was quantified in mg equivalent of citric acid/100 g dry matter.
Quinic acid was quantified in mg equivalent of quinic acid/100 g dry matter.
1-caffeoylquinic acid, 3-O-p-coumaroylquinic acid and 4-caffeoylquinic acid were quantified in mg equivalent of chlorogenic acid/100 g dry matter.
Hydroxytyrosol glucoside and tyrosol glucoside were quantified in mg equivalent of tyrosol/100 g dry matter.
Vanillic acid glucoside was quantified in mg equivalent of hydroxybenzoic acid/100 g dry matter.

[a] Different letters in the same row indicate significant differences ($p < 0.05$).

3.4. Quantification of flavan-3-ols by HPLC-FLD-MS

Fluorimetric detection was used for quantification because it is known to be more selective and provides a stronger signal than MS detection for procyanidins. Calibration curves of (+)-catechin and procyanidin B2 were prepared from 5 to 500 and 1–500 µg/mL, respectively, at 6 concentration levels. Correction factors suggested by Robbins et al. (2009) were used for PCs.

Table 6 shows the quantification results of flavan-3-ols in avocado pulp, peel and seed. Total flavan-3-ols content were 0.152, 19.69 and 16.49 mg/g dry matter in CRA pulp, peel and seed, respectively. These results agree with those reported by Wang et al. (2010). However, total flavan-3-ols content in ORA samples was 19.14%, 16.84% and 12.00% higher than CRA, respectively, for pulp, peel and seed. The increase of PCs during avocado ripening are probably be due to the release of tannins linked to cell-wall structures after softening. The total PCs content in avocado peel and seed were in the range of food commonly known for its high PCs content, such as chocolate and cocoa powder (Gu, House, Wu, Ou, & Prior, 2006).

The compound present in the highest concentration in avocado pulp was catechin/epicatechin with a 46.9% in CRA and 69.1% in ORA pulp. This increase could be due to the breakup of the polymer into smaller moieties. In fact, polymer in CRA represents 25.9% and decrease to 8.5% in ORA pulp.

In avocado peel, there were no significant differences in terms of the percentages of each individual PC between CRA and ORA. It bears highlighting that B-type PCs were predominant in avocado peel. The major PC was the B-type PC dimer with 30.2% and 31.5% in CRA and ORA peel, respectively. It was followed by polymer (24.5% and 23.0% in CRA and ORA peel, respectively) and catechin/epicatechin (18.2% and 17.6% in CRA and ORA peel, respectively).

CRA and ORA seed also presented a similar composition in terms of the percentages of each individual PC. In this avocado by-product, A-type PC dimer, trimer and tetramer together with B-type PC pentamer and hexamer B-type predominated. The major component was the polymer that represented 67.0% and 71.4% in CRA and ORA seed, respectively. The second compound in terms of concentration was catechin/epicatechin, which represented 10.8% and 9.1% in CRA and ORA seed, respectively.

The presence of A-type PCs in avocado peel and seed could provide additional health benefits to these avocado by-products. In fact, A-type PCs have proven to be more resistant to microbial catabolism (Ou et al., 2014) and this could explain why A-type PCs from cranberries have been suggested to prevent urinary-tract infections (Howell et al., 2005).

As far as we are concerned, this is the first time that a quantification of single PCs until DP 13 has been performed in avocado samples. Besides, A and B-type PCs have been quantified separately from DP 2 to 6.

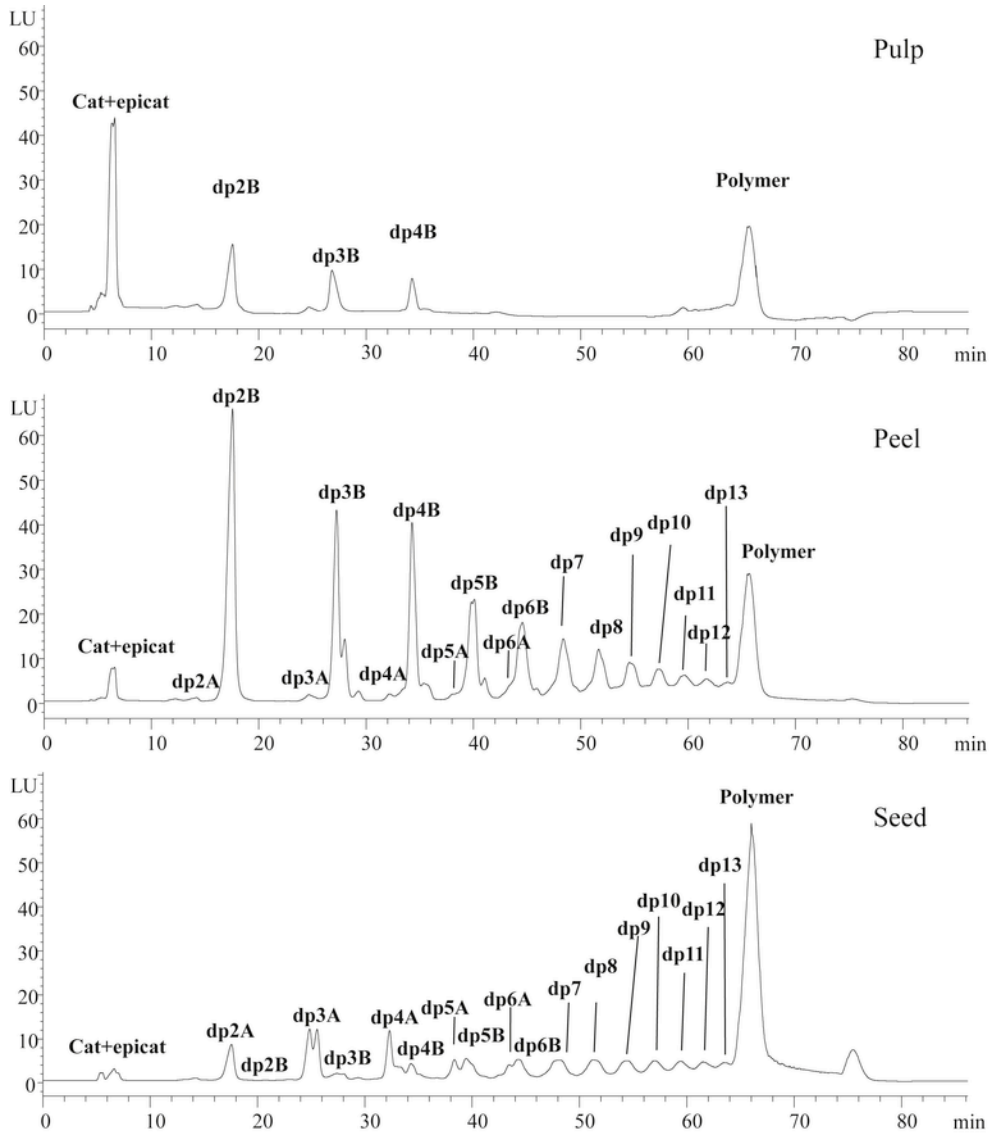

Fig. 1. Fluorescence chromatograms of the identified flavan-3-ols in avocado pulp, peel and seed. (DP: degree of polymerization). Excitation wavelength of 230 nm and emission wavelength of 321 nm.

4. Conclusions

Two different methods were set up with HPLC-DAD-ESI-QTOF-MS to analyze phenolics in avocado fruit and its by-products. For the first time, ESI-QTOF-MS has been applied to the study of these compounds in avocado peel and seed proving to be a valuable tool. Nine compounds in avocado pulp, three in avocado peel and three in avocado seed were identified for the first time using HPLC-DAD-ESI-QTOF-MS. Phenolic compounds quantified with these methods were in higher concentration in ORA pulp and seed than in CRA pulp and seed, probably due to the effect of PAL, which could increase its activity with fruit ripening.

PCs were also determined by HPLC-FLD-MS and catechin/epicatechin, PCs up to DP 13, and the polymers were also identified. It bears emphasizing that this is the first time that PCs up to DP 13 have been quantified singularly, and that A and B-type PCs were differentiated and quantified from DP 2 to 6. The PC content was higher in the three fractions of ORA analyzed than in CRA, possibly because of the hydrolysis of complex tannins.

Finally, the peel and seed from overripe avocado fruit, the main by-products of avocado processing, have demonstrated to possess high concentrations of PCs and they also present A-type PCs. These characteristics make avocado by-products useful matrices to develop functional food, nutraceutical or cosmetics.

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Table 6
Flavan-3-ols in avocado pulp, peel and seed expressed as mg/g dry matter[a].

Compounds	Pulp CRA		Pulp ORA		Peel CRA		Peel ORA		Seed CRA		Seed ORA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
cat + epic	0.071e	0.002	0.130d	0.001	3.58b	0.19	4.16a	0.29	1.77c	0.18	1.70c	0.03
dp2 A					0.96b	0.01	1.01a	0.01	0.94c	0.01	0.93c	0.01
dp2 B	0.016d	0.001	0.015d	0.002	5.94b	0.16	7.46a	0.27	0.23c	0.04	0.23c	0.01
dp3 A					0.12b	0.01	0.12b	0.01	0.65a	0.02	0.68a	0.03
dp3 B	0.018d	0.001	0.019d	0.001	1.41b	0.01	1.67a	0.08	0.19c	0.02	0.17c	0.02
dp4 A					0.121b	0.003	0.15b	0.03	0.34a	0.02	0.31a	0.03
dp4 B	0.0067e	0.0003	0.0079d	0.0001	0.86b	0.02	1.09a	0.06	0.16c	0.01	0.17c	0.01
dp5 A					0.071c	0.003	0.08c	0.02	0.130a	0.001	0.113b	0.001
dp5 B					0.59b	0.03	0.80a	0.03	0.189d	0.004	0.205c	0.003
dp6 A					0.09a	0.01	0.10a	0.01	0.108a	0.005	0.101a	0.008
dp6 B					0.42b	0.02	0.61a	0.04	0.18c	0.01	0.178c	0.001
dp7					0.26b	0.01	0.37a	0.04	0.168d	0.004	0.175c	0.001
dp8					0.178b	0.005	0.25a	0.03	0.138c	0.005	0.139c	0.003
dp9					0.110b	0.001	0.15a	0.02	0.087d	0.001	0.093c	0.001
dp10					0.055b	0.001	0.07a	0.01	0.050c	0.005	0.054b	0.002
dp11					0.043b	0.001	0.06a	0.01	0.044b	0.001	0.045b	0.001
dp12					0.034b	0.002	0.04a	0.01	0.036b	0.002	0.037b	0.001
dp13					0.024b	0.002	0.031a	0.004	0.027a	0.001	0.026a	0.001
polymer	0.0394e	0.0004	0.016f	0.001	4.83d	0.15	5.43c	0.25	11.05b	0.01	13.378a	0.001
total	0.152f	0.003	0.188e	0.001	19.69b	0.57	23.68a	1.20	16.49d	0.22	18.74c	0.06

CRA: optimal consumption ripening avocado; ORA over-ripped avocado.
From DP 3 to polymers were quantified using the corrections factors suggested by Robbins et al. (2009).

[a] Different letters in the same row indicate significant differences ($p < 0.05$).

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