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UHPLC/MS^2-based approach for the comprehensive metabolite profiling of bean (*Vicia faba* L.) by-products: A promising source of bioactive constituents

Ibrahim M. Abu-Reidah [a,b,c,*], David Arráez-Román [a,b], Ismail Warad [c], Alberto Fernández-Gutiérrez [a,b], Antonio Segura-Carretero [a,b]

[a] Analytical Chemistry Department, Faculty of Sciences, University of Granada, Avenida Fuentenueva s/n, 18071 Granada, Spain
[b] Research and Development Center of Functional Food (CIDAF), Health-Sciences Technological Park, Avenida del Conocimiento, 37, 18016 Granada, Spain
[c] Chemistry Department, Faculty of Science, An-Najah National University, P.O. Box. 7, 415 Nablus, Palestine

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ABSTRACT

Today, by-products generated from the agro-industrial practices are considered a key source of bioactive and functional components, that can be used for their nutritional and added value properties. New aspects concerning the use of these wastes as by-products in food production additives or supplements with high nutritional and medicinal value have gained substantial interest, due to their possession of economically high-value products. In this sense, the present study describes a thorough characterization of phytochemical compounds from hydro-methanolic extract of broad beans testa by using ultra-high performance liquid chromatography (UHPLC) hyphenated with quadrupole-time-of-flight tandem mass spectrometry (QTOF-MS). The proposed analytical technique provides tentative characterization of 134 phenolic and other phytochemical compounds in the *Vicia faba* extract, most of which have not been described so far in broad beans. Thus, >85 phytochemicals (Amino acids, phenolic acids, flavonoids, lignans, and terpenoids derivatives) are being reported herein in broad beans pods for the first time. The characterization process was carried out using MS and MS^2 data provided by the ESI-qTOF–MS, along with the use of the relevant literature based on the same botanical family. The data obtained demonstrates that the agro-industrial by-product could potentially be utilized as a promising source of bioactive ingredients to design new functional foods and nutraceuticals with a valuable future market. Furthermore, the obtained data may form a basis for future quantitative and bioavailability studies, which will be the next step in this present work.

1. Introduction

Broad bean (*Vicia faba* L.), is a species of bean family; Fabaceae (Leguminosae), is widely consumed in many countries for its edible seeds. It is a worldwide important crop, native to southwest Asia, North Africa, Europe, and the Middle East countries and extensively cultivated elsewhere. Besides, *Vicia faba* has a long heritage of farming in old world agriculture, being among the most ancient plants in cultivation.

Broad bean is a prized diet component since it contains considerable amounts of valuable nutrients: fiber, lecithin, choline, minerals, and secondary metabolites (Wolosiak et al., 2010). *Vicia faba* is also termed as: fava bean (Faba bean), bell bean, field bean or tic bean. Pulses (legumes) mostly are consumed after a prior industrial process, in which seeds are separated from their pods (testa) by-products to be prepared mainly as fresh, tinned, or frozen food (Carle et al., 2001). Consequently, the big masses of by-products generated during the plant food industry may represent economic and disposal problems due to their high volumes and elimination expenses.

According to FAO estimates, world production of broad beans was estimated to be around 4 million tons (FAOSTAT, 2016). Curiously, harvesting this vegetable would yield around 2.8 million tons of broad bean by-products (~70% of the total production).

A number of by-products have been previously studied as potential sources of antioxidant compounds such as artichoke (Llorach, Espan, Tomas-Barberan, & Ferreres, 2002), olive oil waste waters (Visioli et al., 1999), cocoa (Azizah, Ruslawati, & Swee, 1999), grape seeds and peels (Saura-Calixto, 1998; Larrauri, Sánchez Moreno, & Saura-Calixto, 1998), and potato peel (Rodríguez de Sotillo, Hadley, & Holm, 1994), among others. Actually, added values that can be obtained from such by-products may apply an interesting approach for their use as natural sources of phyto-antioxidant components.

* Corresponding author at: Chemistry Department, Faculty of Science, An-Najah National University, P.O. Box. 7, 415 Nablus, Palestine.
E-mail address: iabureidah@gmail.com (I.M. Abu-Reidah).

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Agro-industrial by-products are considered undervalued matrices because of their removal from foods, besides the problems arise from their treatment and disposal in the ambience (Abu-Reidah, Gil-Izquierdo, Medina, & Ferreres, 2016). Emerging technologies promise to overcome such these problems. According to Galanakis (2012), extraction is regarded the third and most important stage in the recovery of phytochemical antioxidants from plant food by-products. It is necessary to use the potential of new technologies that can reduce extraction time and solvent consumption. Ultrasound-assisted extraction (UAE) method -for example- promises to enhance metabolites extraction process from different food matrices (Roselló-Soto et al., 2015). Indeed, since it is one out of 10 most popular methods applied in the field of food sciences (Galanakis, 2013), being used to increase mass transfer phenomena by cavitation; which promotes biomass diffusion, solvent penetration, and cell disruption and so increases the extraction of phenolics from the food wastes (Barba, Galanakis, Esteve, Frigola, & Vorobiev, 2015).

The recovery of target compounds from food by-products can be achieved by using five distinct recovery stages. This is applied in case of presence of two different ingredients recovered or the valuable component is a micro-molecule (e.g. phenolics). In which, processing progresses from the macroscopic to the macro-molecular level and then to the extraction (or removal) of specific micro-molecules, after that purification and encapsulation is applied for of the target products (Galanakis, 2012).

The active dietary components are most likely contributing to the protective and beneficial effects are the antioxidants (e.g. vitamins, pigments, and polyphenols), which are among the most investigated phytochemicals. Likewise, these antioxidants mainly, one of the phenolic nature are increasingly withdrawing the attention of scientists and industries due to their advantageous effects on human health and food preservation. Nowadays, natural antioxidants are at higher demand due to both consumers' preference and health worries linked with the use of synthetic antioxidants such as BHA, BHT, and Propyl gallate (Augustyniak et al., 2010).

In many studies, *V. faba* was reported as an abundant source of antioxidants phenolic compounds, just like cocoa and chocolates (Okada & Okada, 2007). Recently, it is reported that a great antioxidant activity has been shown by broad bean pods (BBP), also the ingredients of the broad beans extract that decrease oxidative stress in in vivo cells (Mateos-Aparicio, Redondo-Cuenca, & Villanueva-Suárez, 2012).

Among the methods used to determine micro-molecules, it is the high-performance liquid chromatography (HPLC) attached to mass spectrometry (MS^n) detection system, which have been found to be the method of choice in this respect. This instrument also involves tandem MS, which upon its use, more detailed structural information can then be obtained, especially when standard compounds are not commercially available. Earlier studies have established that HPLC–ESI–qTOF-MS^2 is well suited to the non-targeted characterization of plant extracts, giving a wider overview on their phyto-composition (Abu-Reidah, Arráez-Román, et al., 2013).

The broad beans pods (BBP) form an abundant and inexpensive material which has been undervalued yet. Thus, it is important to know the phytochemical composition of those by-products which can be used as a source of natural functional components. In this context, the goal of the present work have been to carry out an extensive metabolite profiling of the methanol/water extract from BBP by using UHPLC-ESI-qTOF-MS^2 as a powerful separation and detection analytical technique.

2. Materials and methods

2.1. Chemicals

All chemicals in the present study were of analytical grade and were used as received. HPLC-grade methanol and acetonitrile were obtained from LabScan (Dublin, Ireland). External standards: (+)-catechin (assay ≥ 97%), apigenin (assay ≥ 99%), sinapic acid (assay ≥ 98%), (-)-epicatechin (assay ≥ 97%), ferulic acid (assay ≥ 99%), myricetin (assay ≥ 98%), luteolin (assay ≥ 97%), quercetin (assay ≥ 95%), apigenin (assay ≥ 99%), carnosic acid (assay ≥ 97%) were purchased from Sigma and Sigma-Aldrich (USA and China). Double-deionized water with a conductivity of <18.0 MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA). Acetic acid of analytical grade (assay > 99.5%) was purchased from Fluka (Buchs, Switzerland)

2.2. Plant material and sample preparation

Fresh commercial whole pods of broad beans (Granadina *cv.*) during their maturation stage were purchased from the local market in Granada (Spain). Thereafter, the deseeded BBP were washed in a clean processing room at 9 °C with distilled water to remove dirt and gritty particles, frozen at −25° C and then lyophilized.

To extract the secondary plant metabolites from BBP, the samples were treated according to the extraction process previously described by Abu-Reidah, Contreras, et al., 2013, with some modifications.

Thus, lyophilized samples (0.5 g) were crushed, turraxed, sonicated (using UAE) with aqueous-methanol (80:20, v/v) in a volumetric flask for 30 min and centrifuged at 4000 × *g* for 15 min, after which the supernatant liquid was collected in a round-bottom flask to be evaporated under vacuum using rotary evaporation at 39 °C. Finally, the dry residue was dissolved with MeOH: H_2O (80:20, v/v), passed through a 0.22 mm syringe filter, and stored at −20 °C for analysis.

2.3. UHPLC conditions

The compounds in BBP extracts were separated on an Agilent HPLC 1200 series (Agilent technologies, Santa Clara, USA) supported with an auto-sampler, vacuum degasser and binary pump. The chromatographic separation was performed in a Zorbax C_{18} analytical column (4.6 mm × 150 mm, 1.8 mm particle size) obtained from Agilent Technologies (Palo Alto, CA, USA). The mobile phases were acetic acid 0.5%, v/v as eluent A, and acetonitrile as eluent B. The chromatographic method comprised the following multistep gradient: 0 min, 0% B, 10 min, 20% B, 30 min, 30% B, 40 min, 50% B, 50 min, 75% B, 60 min, 100% B, 64 min 0% B, and finally a 6 min post-run was used after each analysis. The column temperature kept at 25 °C, and the injection volume was 5 μL. The flow rate was set at 0.80 mL/min throughout the gradient running.

2.4. ESI-qTOF-MS^2 conditions

The UHPLC system was coupled to a Quadrupole-Time-of-Flight (micrOTOF-Q™, Bruker Daltonik GmbH, Bremen, Germany), with a model G1607A ESI interface (Agilent Technologies) that operates in the negative ionization mode. At this step, the use of a T-type splitter was required for coupling with the MS detector. Thus, in this study the flow arrived into the ESI–qTOF–MS detector was 0.2 mL/min. The optimum values of source parameters were as follows: capillary voltage of +4500 V, drying-gas flow, 9 L/min, drying-gas temperature, 190 °C, nebulize ng-gas pressure, 2 bar, collision energy, 10.0 eV, and end-plate offset, −0.5 kV. The source and transfer parameters were optimized to reach the acceptable resolution inside the mass range of the target compounds (50–1100 *m/z*), also to improve the ionization performance.

External MS calibration has been carried out by injecting a sodium acetate solution (5 mM NaOH in water: 2-propanol 1:1 (v/v), with 0.2% of CH_3COOH) in quadratic high precision calibration regression mode. The accurate mass data for the molecular ions were processed through Data Analysis 4.1 software (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formulas by using the Generate Molecular Formula™ editor software.

3. Results and discussion

In the present work, 134 phytochemical compounds (presented in Tables 1, 2 and 3) have been tentatively characterized in the BBP by using the data obtained from ESI-qTOF-MS^2 and related information previously reported in the literature.

An overview of the tentative characterized compounds in BBP extract by UHPLC -ESI-qTOF-MS^2 using negative ion mode is given in Tables 1, 2, and (Table 3, Supplementary material). These compounds are summarized along with their retention time, *m/z* experimental and calculated, mSigma value, tolerance, error (ppm), molecular formula generated by the newer "DataAnalysis 4.1" for the detected molecules, MS^2 fragments and the proposed assignment. All the detected compounds in this work were tentatively characterized by means of their MS data, together with the interpretation of the observed MS^2 spectra in comparison with those found in the literature. The characterization process was based on the latter-mentioned process, since commercial standards were not available for most of compounds detected in this work. UAE treatment has been used as a useful and effective method for the improved recovery of bioactive compounds from beans pods (Roselló-Soto et al., 2015).

Fig. 1 shows the base peak chromatogram (BPC) of the BBP hydromethanolic extract. It is important to note that most of the proposed compounds in this work are reported in BBP for the first time. The structure of several tentatively characterized compounds and the fragmentation pattern, were obtained by the ESI-qTOF-MS in the studied extract, are illustrated in Figs. 2 and 3. An extensive study of the characterized compounds are described below according to their families.

3.1. Organic acids

At the beginning of the BBP chromatogram, four organic acids with low molecular masses could be noticed, namely compounds **1**, **2**, **6** and **30** which were characterized as oxalosuccinic acid, malic acid, citric acid, and hydroxyeucomic acid, respectively (Table 3).

On the other hand, peak **13** (*Rt* 5.24 min), with the molecular mass at *m/z* 243.0628 was assigned to wyerone acid. This compound has been already described in fava beans testa and leaves, and its induced accumulation in tissues was reported to inhibit the fungal growth (Buzi et al., 2003).

3.2. Amino acid and peptide derivatives

Several amino acids (Table 3), have also been detected and characterized including: Vicine (**7**), (iso)leucine (**9**), tyrosine (**12**), and L-tryptophan (**26**), clearly separated, eluting between *Rt* 4.0 and 11.45 min (Fig. 1). The amino acid composition of broad beans seeds has been already described (Abu-Reidah, Arráez-Román, et al., 2013; Abu-Reidah, del Mar Contreras, Arráez-Román, Fernández-Gutiérrez, & Segura-Carretero, 2014). Similarly, the *O*-glycoside non-proteogenic amino acid L-DOPA-glucoside (**8**) gave the major product ion at *m/z* 196.0615. L-DOPA is found to be most effective in the treatment of Parkinson's disease, such glycosides are of growing interest because of their potential facilitated transport through the blood–brain barrier.

At the retention time (*Rt* 5.28 min), the monosaccharide-amino acid, fructose-leucine, was assigned for compound **14**, with molecular mass *m/z* at 292.1404. A newly characterized dipeptide (**19**) has also been detected at *m/z* 229.0830 and it was referred to aspartyl-proline.

3.3. Phenolic compounds and derivatives

Nearly 100 phenolic compounds (Tables 1 and 2), mainly flavonoids and their glycosylated-forms have also been characterized, of which, 72 compounds are reported here in BBP for the first time. This profile qualitatively rich in phenolics, it may partially explain the higher antioxidant activity of broad beans compared with other veggies and legumes.

3.4. Phenolic acids derivatives

Fukiic acid (**17**), with the pseudo-molecular ion at *m/z* 271.0463 amu, and with molecular formula $C_{11}H_{12}O_8$, has been characterized here for the first time in *V. faba*. Likewise, methylfukiic acid (*Rt* 11.82 min) was assigned to compound **28** (Heller & Tamm, 1975). Regarding peak **24**, (*Rt* 9.61 min), having the precursor ion at *m/z* 255.0521 and the fragment ions at *m/z* 193.0504, 179.0349, 165.0555. Thus, **24** has been tentatively identified as piscidic acid.

Other new glycosylated phenolic acids have been detected and characterized in this work. Therefore, compounds **21**, **22**, **25**, and **33** have been tentatively identified as hexosides of homovanillic acid, syringic acid, vanillin, and salicylic acid, respectively. This characterization have been based on the MS and MS^2 fragmentation pattern, which demonstrated the aglycone ions at *m/z* 181.0506, 197.0459, 151.0408 and 137.0227 after having lost a hexose moiety [M–H–162]–.

The two isomers (**34** and **51**), showed the identical molecular formula $C_{16}H_{20}O_9$. In the ESI-qTOF-MS analysis, these isomers exhibited the same fragmentation pattern by losing a hexose moiety (−162 Da) giving rise to a fragment ion at *m/z* 193.0514 as a product ion. Therefore, both compounds were assigned as ferulic acid hexoside isomers. In the same manner, compound **37** (*Rt* 13.94 min) having the pseudo-molecular ion at *m/z* 341.0890, showed a fragment ion at *m/z* 179.0342 (Relative intensity 100%) after losing a hexose moiety (−162 Da), which indicates caffeoyl moiety in structure. According to MS and MS^2 spectral data, **37** was assigned as caffeoylhexose.

The neutral loss of hexose moiety has also been explored in the molecular ion at *m/z* 325.0930 for peaks **29** and **39**, which exhibited the product ions at *m/z* 163.0372 and 119.0496, indicating coumaroyl and the loss of CO_2 (−44 Da), respectively. Both compounds were labelled as coumaroylhexose isomers.

Phenolic acids linked to organic acid (malic acid) was also revealed in the BBP. Three isomers (**45**, **86**, and **90**), showed the molecular ions at *m/z* 279.0509, and intensive fragments corresponding to *p*-coumaric acid residue (*m/z* 163.0673, 119.0475), [M–H–116]–, which implies the loss of malic acid ($C_4H_4O_4$) (Abu-Reidah, Ali-Shtayeh, Jamous, Arráez-Román, & Segura-Carretero, 2015), and relying on the MS and MS^2 spectra and the literature previously reported on the Fabaceae family (Regos, Urbanella, & Treutter, 2009), the isomers were characterized as *p*-coumaric acid-malic acid (Duenas, Hernandez, & Estrella, 2004).

At the retention time 22.50 min, with the molecular ion at *m/z* 309.0616 and the product ion at *m/z* 193.0503 (corresponding to ferulic acid), compound **95** has been tentatively identified as feruloyl-malic acid.

Compound **90** showed an [M–H]– ion at *m/z* 693.2028 in the qTOF-MS (negative-ion), supporting the formula $C_{32}H_{37}O_{17}$ (calc. mass 693.2036). Thus, compound **90** has been suggested as di-*O*-feruloylsucrose.

3.5. Flavonoids derivatives

Glycosylated flavonoids are the most common phenolic compounds found in the BBP (Table 2). Therefore, peak **47**, having the formula $C_{33}H_{40}O_{20}$ and detected main ion at *m/z* 755.2021, has been assigned as quercetin rhamnosyl rutinoside (Abu-Reidah, Contreras, et al., 2013). Compound **73** has been labelled as isoorientin relying on its MS and MS^2 data (Prati et al., 2007).

Peak **41** (*Rt* 15.03 min) with the molecular ion at *m/z* 465.1006 and the formula $C_{21}H_{22}O_{12}$, has shown the product ion at *m/z* 303.0499 (indicates taxifolin), after the neutral loss of hexose moiety (−162 Da) (Fig. 3A). Therefore, it was considered as taxifolin hexoside.

At the retention time 19.73 min, a precursor ion at *m/z* 797.2131 has been detected. This ion had the MS^2 fragment ions at *m/z* 651.1550, 447.0875, 301.0297. Thus, the compound has been tentatively identified as quercetin rhamnopyranosyl-acetylgalactopyranoside-rhamnopyranoside (Spanou et al., 2008). Also, kaempferol-arabinopyranosyl-rhamnopyranoside has been assigned for the

Table 1
Phenolic acids and derivatives detected and characterized in broad beans pods by UHPLC-ESI-qTOF-MS^2.

Peak #	*Rt*	*m/z* Exp.	*m/z* Calc.	Formula	Error (ppm)	mSigma	Major fragments (intensity %)[a]	Assignment	Reference
17	7.53	271.0463	271.0459	$C_{11}H_{12}O_8$	−1.2	2.0	271.0472(19), 195.0304(26), 181.0508(66), 165.0536(9), 151.0392(16), 123.0440(100), 109.0289(58)	Fukiic acid	(Heller & Tamm, 1975)
18	7.83	299.0776	299.0772	$C_{13}H_{16}O_8$	−1.2	1.9	137.0235(100)	Salicylic acid- glucoside	(Abu-Reidah et al., 2014)
20	8.69	315.0717	315.0722	$C_{13}H_{16}O_9$	1.3	5.7	153.0194(49), 152.0102(100), 109.0280(16), 108.0208(56)	Protocatechuic acid hexoside	(Abu-Reidah et al., 2014)
21	8.74	343.1033	343.1035	$C_{15}H_{20}O_9$	0.4	7.6	181.0506(100)	Homovanillic acid hexoside	–
22	9.07	359.0972	359.0972	$C_{15}H_{20}O_{10}$	3.4	6.9	197.0459(100)	Syringic acid hexoside	–
23	9.17	253.0358	253.0354	$C_{11}H_{10}O_7$	−1.6	16.8	165.0562(65), 123.0465(100)	Taraxafolin B	–
24	9.61	255.0521	255.0510	$C_{11}H_{12}O_7$	−4.2	4.0	193.0504(25),179.0349(48), 165.0555(100)	Piscidic acid	(Yang et al., 2013)
25	10.55	313.0928	313.0929	$C_{14}H_{18}O_8$	0.3	13.4	151.0408(69), 107.0511(100)	Vanillin hexoside	–
28	11.82	285.0622	285.0616	$C_{12}H_{14}O_8$	−2.3	2.0	223.0614(34), 209.0462(48), 195.0669(100), 179.0709(10), 163.0403(22), 137.0600(23)	Methylfukiic acid	(Heller and Tamm, 1975)
29	11.91	325.0930	325.0929	$C_{15}H_{18}O_8$	−0.4	19.5	163.0372(64), 119.0496(100)	Coumaroylhexose I	(Abu-Reidah, Arráez-Román, et al., 2013)
33	13.32	299.0791	299.0772	$C_{13}H_{16}O_8$	−6.1	3.3	137.0227(100)	Salicylic acid hexoside	–
34	13.50	355.1034	355.1035	$C_{16}H_{20}O_9$	0.1	3.2	193.0514(100)	Ferulic acid hexoside I	(Reschke & Herrmann, 1982)
35	13.84	305.0662	305.0667	$C_{15}H_{14}O_7$	1.6	25.3	167.0349(34), 137.0242(44), 125.0234(100)	(Epi)gallocatechin	(Abu-Reidah et al., 2014)
36	13.91	457.0970	457.0988	$C_{19}H_{22}O_{13}$	3.9	12.0	341.0873(9), 179.0342(100)	Cutaric acid hexoside	(Fernandez-Pachon, Villano, Troncoso, & Garcia-Parrilla, 2006)
37	13.94	341.0890	341.0878	$C_{15}H_{18}O_9$	−3.6	10.9	179.0342(100)	Caffeoylhexose	–
39	14.80	325.0932	325.0929	$C_{15}H_{18}O_8$	−1.0	4.5	163.0397(100), 119.0497(47)	Coumaroylhexose II	(Winter & Herrmann, 1986)
44	15.38	239.0575	239.0561	$C_{11}H_{12}O_6$	−1.6	1.1	179.0353(100)	Eucomic acid	(Abu-Reidah et al., 2014)
45	15.59	279.0509	279.0510	$C_{13}H_{12}O_7$	0.6	10.3	163.0673(3), 119.0475(5), 93.0557(1)	*p*-Coumaroyl-malic acid I	(Regos et al., 2009)
46	15.87	457.0944	457.0988	$C_{19}H_{22}O_{13}$	1.4	28.9	341.0871(87), 179.0350(100)	Cutaric acid hexoside	(Baxter & Harborne, 1999)
51	16.26	355.1032	355.1035	$C_{16}H_{20}O_9$	0.8	3.5	193.0510(100)	Ferulic acid hexoside II	(Reschke & Herrmann, 1982)
53	16.74	223.0612	223.0612	$C_{11}H_{12}O_5$	−0.2	11.0	119.0495(100)	Sinapic acid[b]	(Yao, Cheng, Wang, Wang, & Ren, 2011)
54	16.76	267.0510	267.0510	$C_{12}H_{12}O_7$	0.0	10.2	181.0491(68), 163.0372(39), 119.0494(100)	Hydroxytrimesic acid trimethyl ester	–
56	17.00	269.0669	269.0667	$C_{12}H_{14}O_7$	−0.8	0.9	209.0455(100),179.0709(45), 148.0527(58)	*O*-Methylpiscidic acid	–
58	17.15	385.1129	385.1140	$C_{17}H_{22}O_{10}$	3.0	6.8	223.0617(100)	Sinapoylhexose	–
65	18.00	295.0456	295.0459	$C_{13}H_{12}O_8$	1.3	4.9	135.0439(26), 133.0156(100), 115.0036(31)	Coutaric acid or Phaseolic acid	–
86	21.36	279.0517	279.0510	$C_{13}H_{12}O_7$	0.6	10.3	163.0396(100), 119.0488(59)	*p*-Coumaroyl-malic acid II	(Regos et al., 2009)
89	21.70	693.2028	693.2036	$C_{32}H_{38}O_{17}$	1.2	32.5	531.1515(100), 337.0885(10), 193.0480(11)	Di-*O*-feruloylsucrose	(Choudhary et al., 2006)
90	21.81	279.0510	279.0510	$C_{13}H_{12}O_7$	0.2	4.1	163.0396(39), 133.0139(100), 119.0503(44)	*p*-Coumaroyl-malic acid III	(Regos et al., 2009)
95	22.50	309.0616	309.0616	$C_{14}H_{14}O_8$	0.1	2.6	193.0503(100)	Feruloyl-malic acid	(Duenas et al., 2004)
98	22.89	193.0507	193.0506	$C_{10}H_{10}O_4$	−0.4	3.7	134.0373(100)	Ferulic acid[b]	–
108	25.17	207.0667	207.0663	$C_{11}H_{12}O_4$	−2.1	5.8	165.0553(85), 147.0442(100), 119.0507(22)	Methylferulic acid	–

[a] Main observed fragments. Other ions were found but they have not been included. The I, II, and III denote isomers having the same MS data.
[b] The identification of compounds has been verified by using authentic standards.

Table 2
Flavonoid glycosides and derivatives from by-products of *Vicia faba*.

Peak #	*Rt*	*m/z* exp.	*m/z* calc.	Formula	Error (ppm)	mSigma	Major fragments (intensity %)[a]	Assignment	Reference
38	14.21	577.1342	577.1351	$C_{30}H_{26}O_{12}$	1.7	20.9	451.1036(21), 425.0873(48), 407.0742(93), 289.0734(100)	Procyanidin B4	(Baxter & Harborne, 1999)
40	14.85	611.1598	611.1618	$C_{27}H_{32}O_{16}$	3.2	6.2	593.1492(9), 521.1302(12), 491.1158(88), 431.0992(12), 401.0869(49), 371.0742(67)	Eriodictyol-di-C-glucoside	(Abu-Reidah et al., 2014)
41	15.03	465.1006	465.1038	$C_{21}H_{22}O_{12}$	7.0	16.3	303.0499(100)	Taxifolin hexoside	–
42	15.07	289.0715	289.0718	$C_{15}H_{14}O_6$	1.0	12.9	245.0828(100), 205.0509(55), 179.0344(56)	Catechin[b]	(Abu-Reidah et al., 2014)
47	15.92	755.2021	755.2040	$C_{33}H_{40}O_{20}$	2.5	9.8	609.1465(2), 301.0322(53)	Quercetin rhamnosylrutinoside	(Fernandez-Pachon et al., 2006)
48	15.96	771.1954	771.1931	$C_{33}H_{40}O_{21}$	4.5	10.9	609.1446(100), 447.0891(11), 463.0741(1)	Hexosylrutin	–
50	16.09	609.1447	609.1461	$C_{27}H_{30}O_{16}$	2.3	10.9	285.0382(35)	Kaempferol di-hexoside	(Knackstedt & Herrmann, 1981)
55	16.95	739.2084	739.2091	$C_{33}H_{40}O_{19}$	0.1	10.6	593.1511(49), 431.0973(2), 269.0684(3)	Rhoifolin glucoside	(Tomas-Barberan et al., 1991)
60	17.53	593.1491	593.1512	$C_{27}H_{30}O_{15}$	3.5	6.9	575.1480(1), 503.1167(1), 473.1072(5), 383.0689(1), 353.0626(1)	Apigenin 6,8-di-C-glucoside (vicenin 2)	(Abu-Reidah et al., 2014)
61	17.55	797.2117	797.2146	$C_{35}H_{42}O_{21}$	3.7	13.2	593.1282(2)	Faralatroside	–
62	17.63	563.1400	563.1406	$C_{26}H_{28}O_{14}$	1.1	14.7	431.0931(21), 269.0525(30)	Apigenin disaccharide	–
63	17.68	597.1797	597.1825	$C_{27}H_{34}O_{15}$	4.7	18.2	435.1351(84), 273.0760(40)	Phloretin di-C-hexoside	–
64	17.87	289.0718	289.0718	$C_{15}H_{14}O_6$	-0.1	5.6	245.0815(100), 205.0507(28), 179.0345(29)	(-)-Epicatechin[b]	(Baxter & Harborne, 1999)
67	18.54	609.1447	609.1461	$C_{27}H_{30}O_{16}$	2.4	2.2	447.0908(12), 301.0353 (1)	Quercetin hexose deoxyhexose	(Abu-Reidah et al., 2014)
68	18.62	755.2018	755.2040	$C_{33}H_{40}O_{20}$	3.0	1.8	609.1463(5), 447.0854(1)	Quercetin rhamnosyl rutinoside	(Kite, Rowe, Lewis, & Veitch, 2011)
70	18.96	725.1919	725.1935	$C_{32}H_{38}O_{19}$	2.2	6.6	285.0254(60)	Kaempferol 3-(2G-xylosylrutinoside)	(Fernandez-Pachon et al., 2006)
71	19.01	609.1447	609.1461	$C_{27}H_{30}O_{16}$	2.4	0.6	463.0867(4), 447.0910(13), 301.0344(1)	Quercetin hexose deoxyhexose III	(Abu-Reidah et al., 2014)
72	19.48	579.1335	579.1355	$C_{26}H_{28}O_{15}$	3.4	6.8	447.0896(12), 301.0315(1)	Quercetin pentose deoxyhexose	(Abu-Reidah et al., 2014)
73	19.63	447.0909	447.0933	$C_{21}H_{20}O_{11}$	5.3	11.4	429.0839(19), 387.0647(3), 357.0603(100), 327.0536(95)	Isoorientin	(Prati et al., 2007)
74	19.73	797.2131	797.2146	$C_{35}H_{42}O_{21}$	1.9	2.6	651.1550(2),447.0875 (1), 301.0297(1)	Quercetin-rhamnosyl acetyl-hexoside-rhamnoside	(Spanou et al., 2008)
75	19.77	781.2181	781.2197	$C_{35}H_{42}O_{20}$	1.8	13.0	635.1625(38)	Kaempferol 3-rhamnosyl-acetyl-galactoside-rhamnoside I	(Tomas-Barberan et al., 1991)
76	19.88	593.1499	593.1512	$C_{27}H_{30}O_{15}$	2.2	2.9	447.0921(46), 285.0398(2)	Kaempferol-hexoside-rhamnoside I	(Tomas-Barberan et al., 1991)
77	19.90	739.2081	739.2091	$C_{33}H_{40}O_{19}$	1.4	4.7	593.1493(72), 447.0941(1), 285.0392(1)	Kaempferol-rhamnosyl-galactoside-rhamnoside	(Tomas-Barberan et al., 1991)
78	19.97	465.1383	465.1402	$C_{22}H_{26}O_{11}$	4.2	29.9	303.0875(100), 179.0351(18)	Methylepicatechin hexoside	–
79	20.03	641.1367	641.1359	$C_{27}H_{30}O_{18}$	-1.3	14.5	479.0771(100), 317.0273(9)	Myricetin di-hexoside	(Abu-Reidah et al., 2014)
80	20.09	709.1965	709.1985	$C_{32}H_{38}O_{18}$	2.9	3.1	563.1380(39), 431.0952(2), 285.0248(1)	Kaempferol-xylo-pyranosyl-rhamnoside-rhamnoside	(Gamal-Eldeen, Kawashty, Ibrahim, Shabana, & El-Negoumy, 2004)
82	20.56	781.2192	781.2197	$C_{35}H_{42}O_{20}$	0.5	2.4	635.1605(45), 431.0953(1), 285.0450(1)	Kaempferol-(rhamnosyl-acetyl-galactoside)-rhamnoside II	(Tomas-Barberan et al., 1991)
83	20.69	563.1390	563.1406	$C_{26}H_{28}O_{14}$	2.9	3.3	417.0805(11), 285.0415(2)	Kaempferol-arabinosyl-rhamnoside	(Tselepi et al., 2011)
84	20.82	593.1497	593.1512	$C_{27}H_{30}O_{15}$	2.5	2.7	447.0907(22), 301.0361(2)	Quercetin di-rhamnoside	–
85	21.26	651.1559	651.1567	$C_{29}H_{32}O_{17}$	1.2	6.7	609.1478(1), 447.0892(7), 301.0360(1)	Quercetin-acetyl-rutinoside II	(Tomas-Barberan et al., 1991)
87	21.51	797.2121	797.2146	$C_{35}H_{42}O_{21}$	3.1	26.7	593.1441(12), 489.1031(100), 285.0393(5)	Kaempferol-rhamnosyl-galactoside-rhamnoside	(Spanou et al., 2008)
91	21.92	431.0972	431.0984	$C_{21}H_{20}O_{10}$	2.8	11.4	413.0858(3), 371.0754(1), 341.0661(29), 311.0559(100), 269.0451(1)	Apigenin 8-C-glucoside (Isovitexin)	(Abu-Reidah et al., 2014)
92	22.15	625.1410	625.1410	$C_{27}H_{30}O_{17}$	0.0	23.1	463.0871(100)	Quercetin di-hexoside	(Baxter & Harborne, 1999)
93	22.22	463.0864	463.0882	$C_{21}H_{20}O_{12}$	4.0	12.1	317.0296(45)	Myricetin rhamnoside	–
94	22.30	641.1355	641.1359	$C_{27}H_{30}O_{18}$	0.7	13.0	479.0796(100), 317.0297(3)	Myricetin di-hexoside II	(Abu-Reidah et al., 2014)
96	22.55	577.1548	577.1563	$C_{27}H_{30}O_{14}$	2.5	3.2	431.0969(83), 285.0401(11)	Kaempferol di-rhamnoside	(Tselepi et al., 2011)
97	22.75	635.1631	635.1618	$C_{29}H_{32}O_{16}$	-2.0	51.3	593.1441(1),431.0934 (11), 285.0392(2)	Kaempferol-acetyl glucoside-rhamnoside	(Tomas-Barberan et al., 1991)
100	23.02	435.1369	435.1297	$C_{21}H_{24}O_{10}$	3.9	16.8	273.0802(63)	Phloretin glucoside	(Escarpa & González, 2000)
101	23.29	635.1604	635.1618	$C_{29}H_{32}O_{16}$	2.1	10.0	593.1500(1), 489.1028 (9), 431.0926(7), 285.0396(1)	Kaempferol-acetyl-glucoside-rhamnoside	(Tomas-Barberan et al., 1991)
103	23.61	533.1687	533.1664	$C_{26}H_{30}O_{12}$	-4.3	11.9	371.1061(90)	Noricaritin hexoside	–
105	24.17	447.0928	447.0933	$C_{21}H_{20}O_{11}$	1.0	4.9	284.0339(61), 285.0377(30)	Kaempferol 3- or 7-glucoside	(Tomas-Barberan et al., 1991)
106	24.23	433.0729	433.0729	$C_{20}H_{18}O_{11}$	2.6	39.9	301.0297(38), 300.0284(100)	Quercetin-arabinofuranoside	(Kim et al., 2011)
107	25.11	447.0916	447.0933	$C_{21}H_{20}O_{11}$	3.9	13.0	301.0345(95)	Quercetin rhamnoside	(Saber et al., 1998)
109	25.44	609.1801	609.1825	$C_{28}H_{34}O_{15}$	3.9	17.6	301.0728(100)	7-rutinoside or 7-neohesperidoside of hesperetin	–
110	25.73	463.0874	463.0882	$C_{21}H_{20}O_{12}$	1.8	22.7	301.0355(100)	Quercetin hexoside	(Onyilagha et al., 2009)

(continued on next page)

Table 2 (*continued*)

Peak #	*Rt*	*m/z* exp.	*m/z* calc.	Formula	Error (ppm)	mSigma	Major fragments (intensity %)[a]	Assignment	Reference
111	25.86	269.0448	269.0455	$C_{15}H_{10}O_5$	−2.9	19.1	153.0220(11), 133.0281(38)	Trihydroxyl-flavone	(Tomas-Barberan et al., 1991)
112	26.08	593.1509	593.1512	$C_{27}H_{30}O_{15}$	0.5	37.6	447.0808(6), 285.0254(10)	Kaempferol-hexoside-rhamnoside	(Hargreaves & Mansfield, 1975; Baxter & Harborne, 1999)
113	26.11	273.0776	273.0768	$C_{15}H_{14}O_5$	−2.8	44.3	nd	Phloretin	–
115	26.45	711.2157	711.2142	$C_{32}H_{40}O_{18}$	−2.1	12.2	505.1681(100), 463.1613(64), 301.1078(82)	Homoeriodictyol-(hydroxybutanoyl) hexosyl-hexoside	–
116	26.85	479.0804	479.0831	$C_{21}H_{20}O_{13}$	4.5	8.4	317.0292(100)	Myricetin-*O*-hexoside	(Guajardo-Flores et al., 2012)
117	27.20	477.1418	477.1402	$C_{23}H_{26}O_{11}$	−3.2	27.9	317.0235(67)	Myrciacitrin I	–
119	27.51	317.0289	317.0303	$C_{15}H_{10}O_8$	4.3	6.7	287.0193(100), 178.9983(34)	Myricetin[b]	(Herrmann & Wöldecke, 1977)
120	27.64	431.0938	431.0984	$C_{21}H_{20}O_{10}$	10.6	38.2	285.0371(100)	Kaempferol deoxyhexose	(Abu-Reidah et al., 2014)
122	27.84	447.0917	447.0933	$C_{21}H_{20}O_{11}$	3.5	8.4	301.0330(99)	Quercetin deoxyhexose	(Abu-Reidah et al., 2014)
123	28.31	489.1022	489.1038	$C_{23}H_{22}O_{12}$	3.3	40.0	285.0402(22)	Kaempferol-acetyl-hexoside	(Prati et al., 2007)
124	28.58	579.2075	579.2083	$C_{28}H_{36}O_{13}$	−3.1	37.9	517.3078(4), 385.1481(23), 223.0945(9), 193.0508(100)	Neohesperidin di-hydrochalcone	–
125	28.80	577.1524	577.1563	$C_{27}H_{30}O_{14}$	6.8	15.9	433.1137(20), 271.0616(100)	Pelargonidin rutinoside	–
126	29.05	253.0502	253.0506	$C_{15}H_{10}O_4$	1.5	32.5	153.0171(8), 135.0102(13), 117.0342(12)	Dihydroxyflavone	(Tomas-Barberan et al., 1991)
129	31.27	431.0963	431.0984	$C_{21}H_{20}O_{10}$	4.8	24.1	285.0399(100)	Kaempferol-rhamnoside	(Abu-Reidah et al., 2014)
131	32.22	285.0399	285.0405	$C_{15}H_{10}O_6$	1.8	13.2	217.0474(4), 151.0024(4), 133.0303(3)	Luteolin[b]	(Tomas-Barberan et al., 1991)
132	32.46	607.2016	607.2032	$C_{29}H_{36}O_{14}$	2.6	40.3	463.1551(58), 301.1094(100)	Quercetin derivative	–
133	32.49	301.0342	301.0354	$C_{15}H_{10}O_7$	0.5	15.6	179.0036(63), 151.0013(100)	Quercetin[b]	–
134	32.56	505.1700	505.1751	$C_{25}H_{30}O_{11}$	3.1	31.4	301.1085(100), 135.0453(81)	Dimethoxy-pterocarpan-hexo-acetate	(Tan et al., 2012)
137	34.96	271.0627	271.0612	$C_{15}H_{12}O_5$	−5.6	13.3	153.0181(42), 135.0389(100)	Naringenin	(Aguilera, Estrella, Benitez, Esteban, & Martín-Cabrejas, 2011)
139	35.55	269.0456	269.0455	$C_{15}H_{10}O_5$	0.0	9.5	151.0038(8)	Apigenin[b]	–
141	36.08	299.0552	299.0561	$C_{16}H_{12}O_6$	3.0	116.0	284.0328(100)	Chrysoeriol or diosmetin	–
142	42.28	283.0613	283.0612	$C_{16}H_{12}O_5$	−0.3	7.1	268.0381(100)	Geraldone	(Tomas-Barberan et al., 1991)

[a] Main observed fragments. Other ions were found but they have not been included. The I, II, and III denote isomers having the same MS data.
[b] The identification of compounds has been verified by using authentic standards.

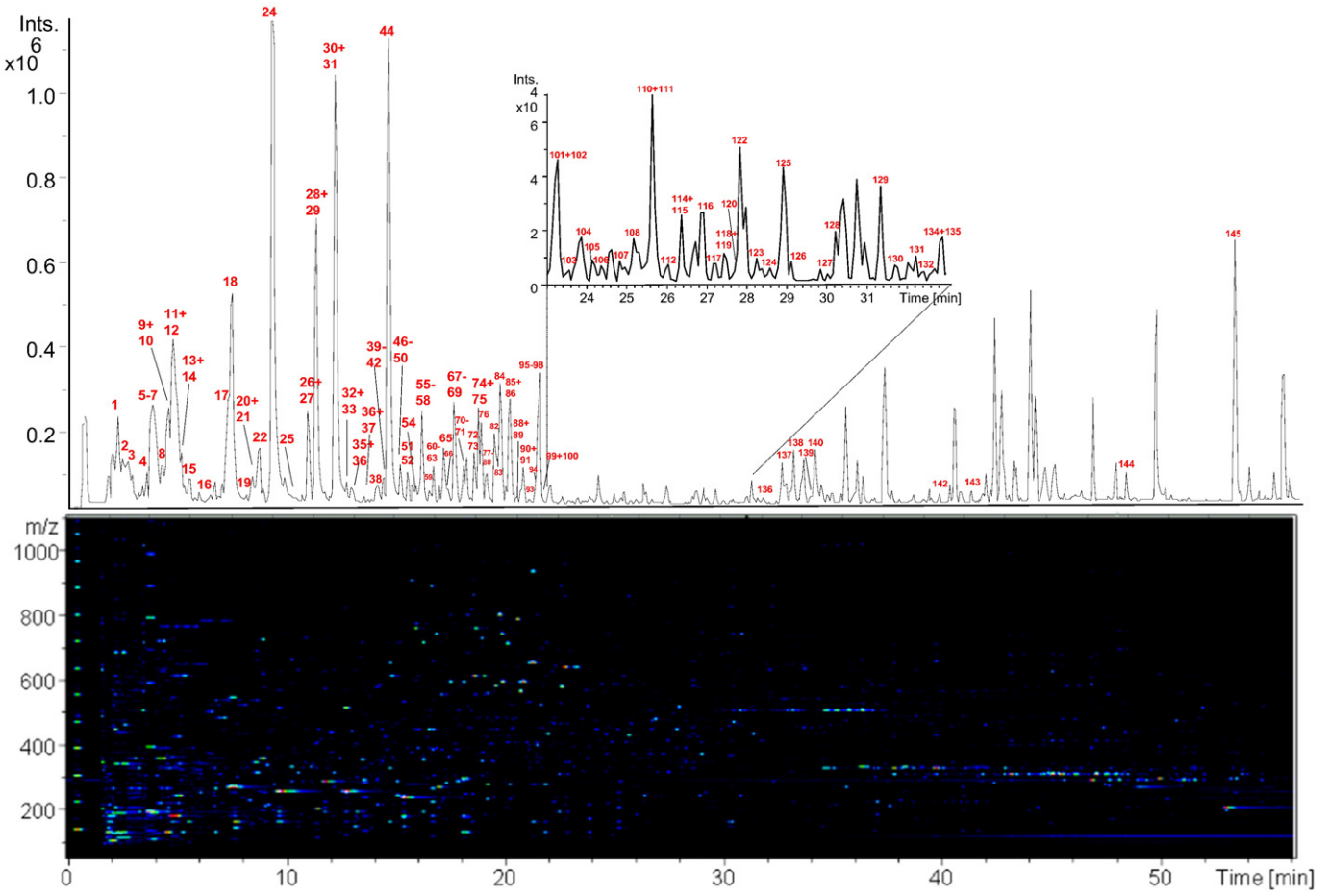

Fig. 1. Base peak chromatogram (BPC) together with Density view window of broad beans pods by-products.

pseudo-molecular ion at *m/z* 563.1390, depending on the qTOF-MS data and as previously reported (Tselepi et al., 2011).

On the other hand, compound **84** (*Rt* 20.82 min) with the molecular formula $C_{27}H_{30}O_{15}$, has the fragment ions at *m/z* 447.0907 and 301.0361. Thus, this compound was tentatively characterized as quercetin di-rhamnoside. The compound detected at the retention time 21.92 min (**91**), having the main ion at *m/z* 431.0972 and the fragment ions at *m/z* 413.0858, 371.0754, 341.0661, 311.0559, and 269.0451, characteristic of *C*-glycosidic fragmentation pattern, and so it was identified as apigenin 8-*C*-glucoside (isovitexin) (Abu-Reidah et al., 2014).

With the pseudo-molecular ion at *m/z* 463.0864 and the formula $C_{21}H_{20}O_{12}$, a fragment ion at *m/z* 317.0296 has appeared (indicating myricetin in structure), after the neutral loss of a rhamnose moiety, thus, the main ion has been characterized as myricetin rhamnoside.

Compound **100** showed the molecular ion at *m/z* 435.1369 and the fragment ion at *m/z* 273.0802 (referred as to phloretin in structure), arose after a loss of a glucose moiety. Depending on the data of the MS, the MS^2 fragmentation pattern, and as previously reported (Escarpa & González, 2000), this compound could be assigned as phloretin glucoside (Fig. 3B). With the precursor ion at *m/z* 533.1687 (**103**), and the fragment ion at *m/z* 371.1061 appeared after the neutral loss of a hexose moiety. Accordingly, **103** was assigned to noricaritin hexoside. Also, compounds **106** and **107** had the fragment ion at *m/z* 301.03, which corresponds to quercetin in structure, and have been tentatively labelled as quercetin-arabinofuranoside and quercetin rhamnoside, respectively (Saber, Abou-Zeid, & Barakat, 1998; Kim et al., 2011).

At the retention time 25.44 min, the precursor ion at *m/z* 609.1801 and the fragment ion at *m/z* 301.0728 (hesperetin), were appeared in the ESI-qTOF-MS analysis. This compound was characterized as 7-rutinoside or 7-neohesperidoside of hesperetin. On the other hand, the fragment ion in the MS^2 analysis detected at *m/z* 301.0355 (100% relative intensity) corresponded to quercetin in structure. Thus, the main ion was assigned as quercetin hexoside (Fig. 3C) (Onyilagha, Islam, & Ntamatungiro, 2009).

The pseudo-molecular ion at *m/z* 711.2157 ($C_{32}H_{40}O_{18}$), has been tentatively proposed as homoeriodictyol-(hydroxybutanoyl) hexosyl-hexoside, relying on the data of MS and MS^2 spectral data.

The compound (*Rt* 26.85) with the molecular ion at *m/z* 479.0804 has shown the fragment ion at *m/z* 317.0292 (represents myricetin). Based on the MS, MS^2 data and as previously reported (Guajardo-Flores, García-Patiño, Serna-Guerrero, Gutiérrez-Uribe, & Serna-Saldívar, 2012), the compound was characterized as myricetin hexoside. In the same way, compound **117** has been labelled as myrciacitrin I.

At the retention time 22.55 min, a peak with the molecular formula $C_{27}H_{30}O_{14}$ was detected at *m/z* 577.1548. The MS^2 spectrum has shown two fragment ions at *m/z* 431.0969 [M-H-146]- and 285.0401 [M-H-146]-, denoting two sequent losses of rhamnose moiety. Therefore, compound **96** was tentatively identified as kaempferol di-rhamnoside (Choudhary, Begum, Abbaskhan, Shafiq ur, & Atta ur, 2006). Likewise, compound **84** was assigned as quercetin di-rhamnoside based on its correct and acceptable data obtained from ESI-qTOF.

Signal **123** has shown the exact mass at *m/z* 489.1022 and the fragment ion at *m/z* 285.0402 (kaempferol in structure) after losing (−204 Da) which is a typical fragment for actyl-hexose moiety. Thus, the compound has been assigned as kaempferol-acetyl glucoside, an already detected compound in forage and legumes (Prati et al., 2007). At the retention time 32.46 min, the molecular ion at *m/z* 607.2016 had exhibited the fragment ions at *m/z* 463.1551 and 301.1094. Based on data from ESI-qTOF, the compound was proposed as a derivative of quercetin. Finally, peak **142** with the molecular ion at *m/z* 283.0613 has

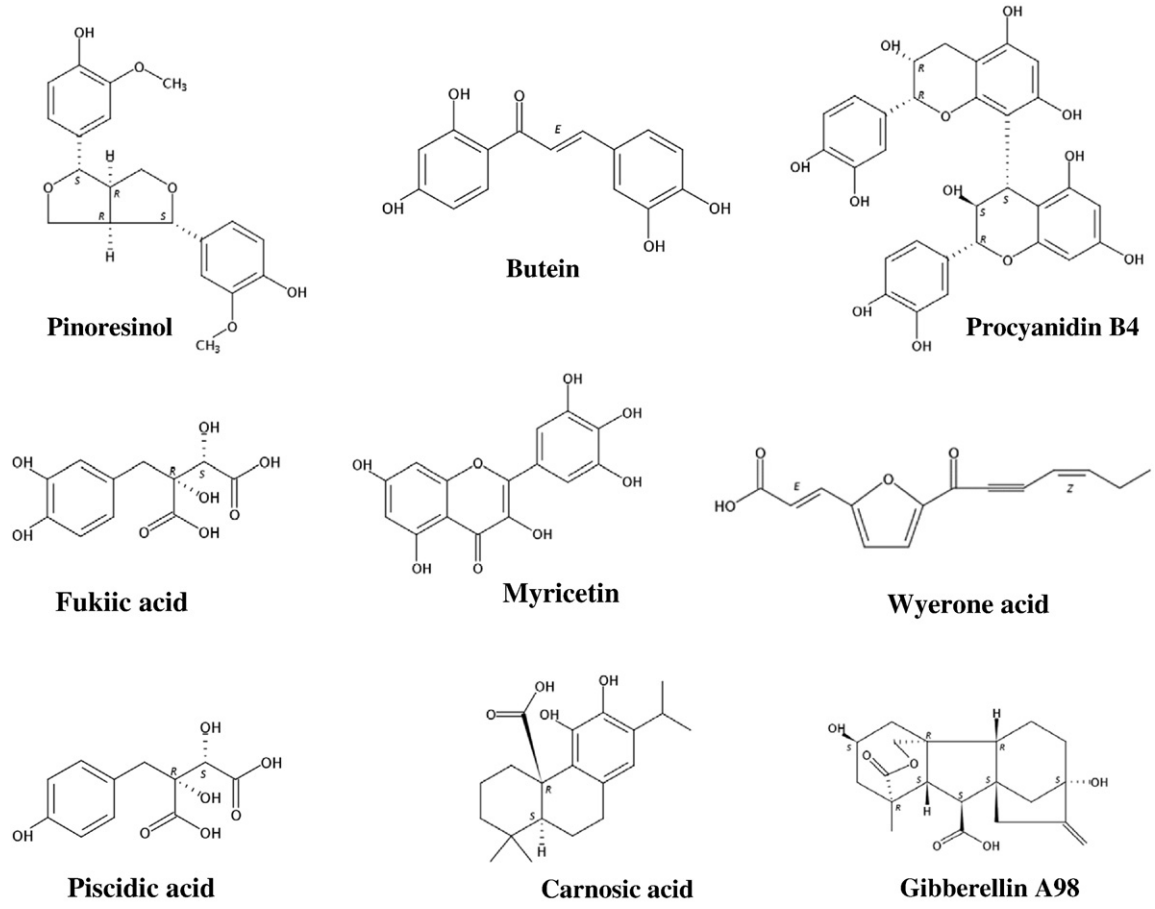

Fig. 2. Structure of some phytochemicals characterized in broad bean pods.

shown the fragment ion at *m/z* 268.0381(100%), after a neutral loss of 15 Da (-CH_3). Thus, this compound was assigned as geraldone (Tomas-Barberan, Garcia-Grau, & Tomas-Lorente, 1991).

3.6. Lignan derivatives

Eight lignan derivatives have been identified in the hydro-methanolic extract of BBP in this study. Interestingly, these lignan derivatives (see Table 1) are being reported here in fava beans for the first time. Thus, two glycosylated lignans (**57** and **69**) showed the molecular ions at *m/z* 551.1743 and the identical formula $C_{26}H_{32}O_{13}$. Both compounds have been labelled as prinsepiol hexoside isomers. The fragment ion at *m/z* 389.1228 (indicating prinsepiol in structure), has appeared after the neutral loss of a hexose moiety (Fig. 3D). In the same manner, compounds **81** and **88** were identified as (iso)lariciresinol hexoside isomers. On other hand, the pseudo-molecular ion at *m/z* 519.1860 at the retention time 23.75 min, has displayed the fragment ion at *m/z* 357.1336 (referred to pinoresinol), which appeared after a neutral loss of hexose moiety. Last, compounds **130** and **135** have been characterized as lyoniside and pinoresinol (Holse, Husted, & Hansen, 2010), respectively.

Otherwise, compound **104** (*Rt* 23.75 min) exhibited the molecular ion [M-H]- at *m/z* 519.1860 and the fragment ion at *m/z* 357.1336(100) (indicating pinoresinol in structure), and was labelled as pinoresinol hexoside.

3.7. Other phytochemicals

Besides the major categories of phytochemicals, in the pods extract analyzed, other phytochemicals belong to different families of compounds have also been detected.

Thus, compound **38** detected at 14.21 min, with molecular formula $C_{30}H_{26}O_{12}$ has been tentatively identified as procyanidin B4. (Baxter & Harborne, 1999).

Compound **121** and **125** were assigned as cowanol and pelargonidin rutinoside (Fig. 3E), respectively. Compound **128** detected at 30.21 min, had the molecular ion at *m/z* 163.0762 and showed the fragment ion at *m/z* 148.0541, has been tentatively suggested as eugenic acid. While, the precursor ion at *m/z* 331.1913 had a fragment ion at *m/z* 287.2009. Then, the parent ion was assigned as carnosic acid.

Finally, two other terpenoids, namely, gibberellin A29 and gibberellin A98, have been detected and proposed for the compounds **52** and **127**, respectively. These compounds have already been noticed in the immature seeds of broad beans (Sponsel, Gaskin, & MacMillan, 1979), but not in the pods.

4. Conclusion

In the present work, the HPLC-ESI-qTOF-MS^2 based qualitative analysis of the phytochemicals from BBP, provided high resolution and mass accuracy which allowed the feasibility of the phyto-components untargeted characterization which was based on MS and MS/MS spectra in negative ion mode, together with the related data from the literature. The use of the proposed method is helpful to detect and to characterize 134 phenolic compounds, thereof, 85 were identified herein in BBP for the first time. This study highlights BBP matrix as a rich source of bioactive compounds. The analysis of the BBP revealed larger number of compounds, most being glycosylated derivatives of flavonoids and phenolic acids. Subsequently, the presence of this large quality of dietary phenolics and other polar components provide significant added-value to these by-products, as well, encourage the use of the BBP extract as a

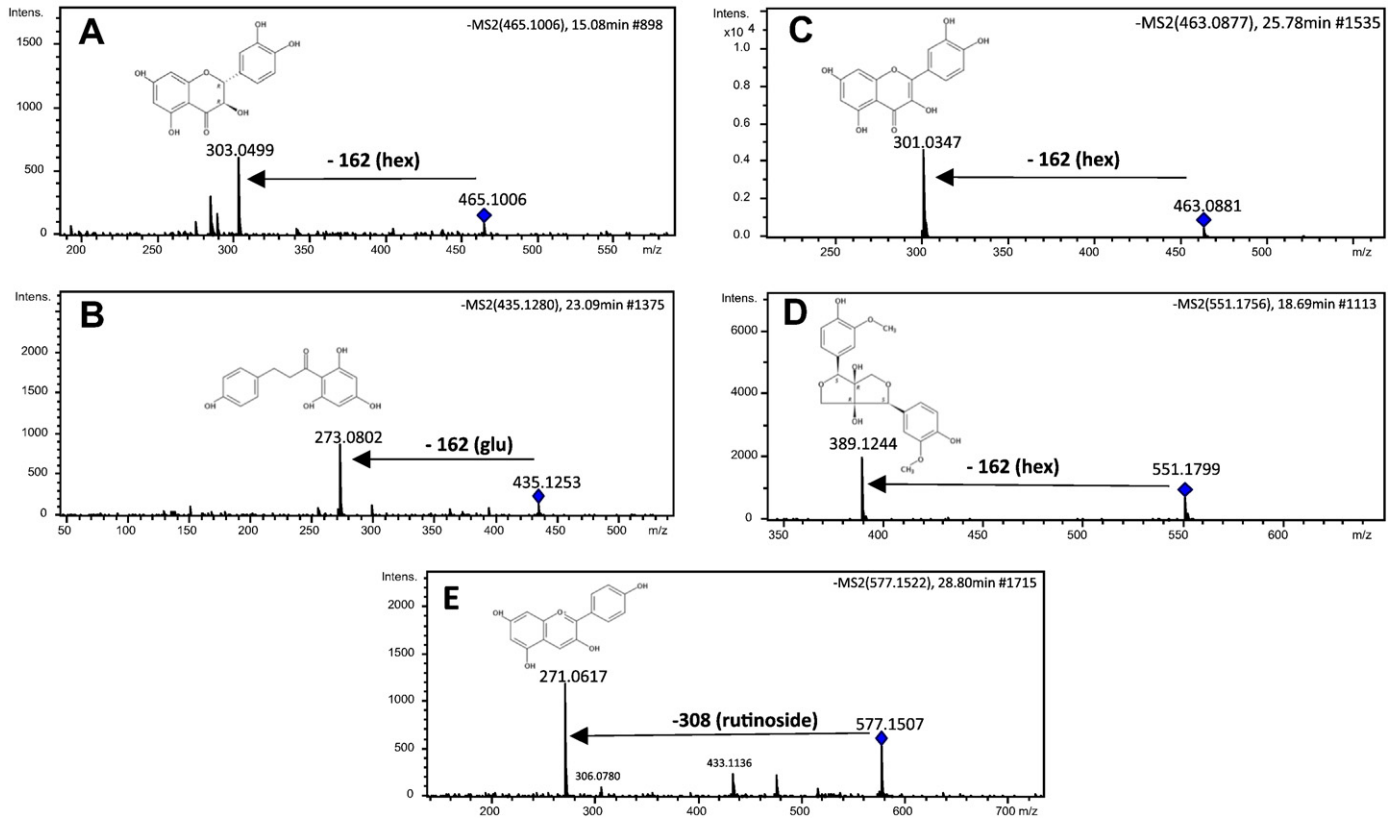

Fig. 3. Fragmentation pattern of some newly detected and identified compounds; A. taxifolin hexoside, B. phloretin glucoside, C. quercetin hexoside, D. prinsepiol hexoside, and E. pelargonidin rutinoside.

source of functional ingredients for the development of added value products to boost health. Moreover, the data compiled may boost further use of this agri-food by-product matrices in pharmaceutical, nutraceutical, and cosmetic applications.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2017.01.014.

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