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First report of non-coloured flavonoids in *Echium plantagineum* bee pollen: differentiation of isomers by liquid chromatography/ion trap mass spectrometry

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Apicultural products have been widely used in diet complements as well as in phytotherapy. Bee pollen from *Echium plantagineum* was analysed by high-performance liquid chromatography/photodiode-array detection coupled to ion trap mass spectrometry (HPLC-PAD-MS^n) with an electrospray ionisation interface. The structures have been determined by the study of the ion mass fragmentation, which characterises the interglycosidic linkage in glycosylated flavonoids and differentiates positional isomers. Twelve non-coloured flavonoids were characterised, being kaempferol-3-*O*-neohesperidoside the major compound, besides others in trace amounts. These include quercetin, kaempferol and isorhamnetin glycosides, with several of them being isomers. Acetylated derivatives are also described. This is the first time that non-coloured flavonoids are reported from this pollen, with MS fragmentation proving to be most useful in the elucidation of isomeric structures. Copyright © 2010 John Wiley & Sons, Ltd.

Bee pollen is collected from various selected flower species by the honeybee *Apis mellifera* and is the only natural source of proteins, lipids, vitamins, minerals and amino acids that are essential for the growth and development of bees.[1] The bees consume pollen in their own diets and use it to feed larvae. Bee pollen is also used as a dietary supplement for humans and several works focus on the antioxidant activity of bee collected pollen and its total phenolics.[2,3] The colour, size and morphology of each pollen pellet vary in relation to the source of the species.[1,4]

Among bee pollen components, a profile of flavonoids and anthocyanins has been used in order to establish quality parameters of the pollen, to characterise it in terms of botanical origin, recognise taxonomic markers and to evaluate their nutritional and biological properties.[1]

Echium plantagineum (Boraginaceae) is an erect annual (occasionally biennial) species, commonly 30–60 cm in height that reproduces by seed. It is commonly known as Patterson's Curse and is native to southern Europe.[5] *E. plantagineum* bee pollen has been extensively studied concerning a number of characteristics, including amino acids,[6] pyrrolizidine alkaloids and their *N*-oxides,[7,8] among others. However, no studies regarding the phenolic composition, besides anthocyanins,[1] are available.

In the phytochemical analysis of flavonoid extracts, the occurrence of complex mixtures is very frequent and the isolation of the different compounds prior to the identification is a very difficult task. High-performance liquid chromatography/multi-stage mass spectrometry (HPLC/MS^n) analysis provides a method of choice for the characterisation of complex flavonoid molecules in these extracts without isolating and purifying.[9] In fact, the use of HPLC coupled with MS is extremely valuable as a screening tool where the scarcity of source material prevents the ready isolation and purification of compounds for later identification, and especially for the analysis of challenging metabolites and for the differentiation of isomers.[9,10] This technique has been successfully applied in the characterisation of complex extracts, even when some compounds are present in trace amounts.[11]

The aim of this work was to study non-coloured flavonoids in the bee pollen from *E. plantagineum* for the first time, using liquid chromatography-photodiode-array detection coupled to ion trap mass spectrometry (HPLC-PAD-MS^n) with an electrospray ionisation (ESI) interface. This technique revealed to be capable of differentiation of positional isomers and the results add to the knowledge on bee pollen chemistry, which is important as quality control marker and constitutes the first step to understand the several biological activities displayed by this material.

EXPERIMENTAL

Chemicals and reagents

Methanol and formic acid were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

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Sampling

Bee pollen sample was provided by bee-keepers in Lousã (central Portugal). Botanical origin was assured by Prof. Paula B. Andrade, PhD (Department of Pharmacognosy, Faculty of Pharmacy, Porto University).

Extraction

Bee pollen (0.2 g) from the *E. plantagineum* sample was thoroughly mixed with 1 mL methanol/water (5% formic acid) (7:3), ultra-sonicated for 18 h, centrifuged at 12 000 *g* and filtered through a 0.45 μm pore filter.

HPLC-PAD-MS^n ESI

Chromatographic analysis were carried out on a LiChroCART column (250 × 4 mm, RP-18, 5 μm particle size, LiChrospher®100 stationary phase; Merck, Darmstadt, Germany) protected with a LiChroCART guard column (4 × 4 mm, RP-18, 5 μm particle size; Merck, Darmstadt, Germany). The mobile phase consisted of two solvents: water/formic acid (1%) (A) and methanol (B). Elution started with 30% B and a gradient was used to obtain 40% at 20 min, 50% at 25 min, 60% at 30 min and 80% at 32 min. The flow rate was 1 mL/min, and the injection volume 20 μL. Spectral data from all peaks were accumulated in the range 240–600 nm, and chromatograms were recorded at 350 nm for flavonoids and at 520 nm for anthocyanins. The HPLC-PAD-MS^n ESI analysis were carried out in an Agilent HPLC 1100 series equipped with a diode-array detector and mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC system consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a photodiode array detector (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, v. 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an ESI interface and was controlled by LCMSD software (Agilent, v. 4.1). The ionisation conditions were adjusted to 350°C and 4 kV for capillary temperature and voltage, respectively. The nebuliser pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full scan mass covered the range from *m/z* 100 up to *m/z* 2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative ionisation mode for flavonoids and positive ionisation mode for anthocyanins. MS^n was carried out in the automatic mode on the more abundant fragment ion in MS^{n-1}, and in the manual mode for MS^3[739→593]$^-$ of compound **5**.

The classical nomenclature[12] for glycoconjugates was adopted to designate the fragment ions. Ions $^{k,l}X_j$, Y^n_j, Z^n_j represent those fragments still containing the flavonoid aglycone, where *j* is the number of the interglycosidic bond broken, counted from the aglycone, *n* represents the position of the phenolic hydroxyl where the oligosaccharide is attached, and *k* and *l* denote the cleavage within the carbohydrate rings.

The ions obtained as a consequence of a second oligosaccharide fragmentation have been labelled according to previous reports.[13] Thus, ions obtained from the ion $[Y^3_{2''}]^-$ (-MS^3[(M-H)→$Y^3_{2''}$]$^-$) (Figs. 4 and 5) have been labelled starting with the ion $[Y^3_{2''}]^-$ and followed by the resultant MS^3 ion, e.g. the ion $[Y^3_{2''}{}^{0,3}X^3_0]^-$ originated from the fragmentation of ion $[Y^3_{2''}]^-$ (loss of rhamnose in the 2″ position) and denotes the loss of the glycosidic fraction that involves carbons 4″-6″ produced by the internal cleavage of the hexoses (Fig. 5). The losses indicated in the MS^3 scan show that the fragment came from the trapped and fragmented ion $[Y^3_{2''}]^-$ and not from the deprotonated molecular ion.

RESULTS AND DISCUSSION

The HPLC-PAD-MS^n ESI study of the hydro-alcoholic extract of bee pollen from *E. plantagineum* revealed a number of non-coloured flavonoids (Fig. 1), of which the most abundant was

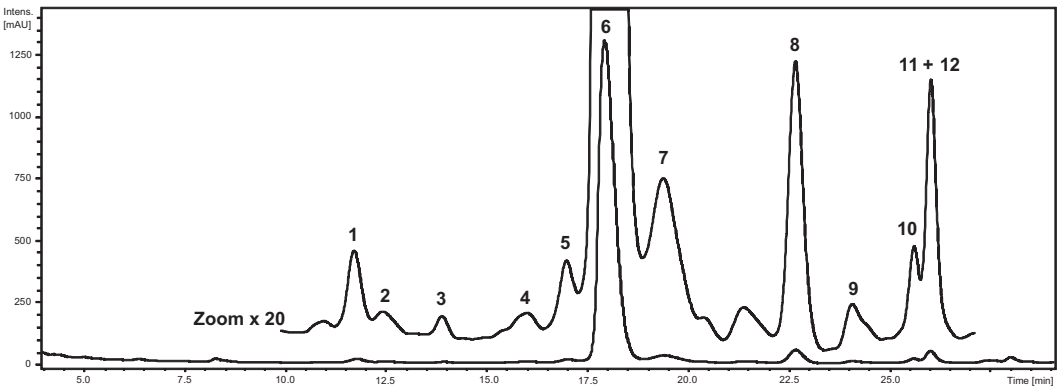

Figure 1. HPLC-PAD (350 nm) phenolic profile from bee pollen of *Echium plantagineum.* Identity of peaks: quercetin-3-*O*-sophoroside (**1**), isorhamnetin-3-*O*-rutinoside (**2**), quercetin-3-*O*-neohesperidoside (**3**), kaempferol-3-*O*-sophoroside (**4**), kaempferol-3-*O*-(4″-rhamnosyl)neohesperidoside (**5**), kaempferol-3-*O*-neohesperidoside (**6**), kaempferol-3-*O*-neohesperidoside derivative (**7**), kaempferol-3-*O*-(3″/4″-acetyl)neohesperidoside (**8**), kaempferol-3-*O*-neohesperidoside-7-O-rhamnoside (**9**), kaempferol-3-*O*-glucoside (**10**), kaempferol-3-*O*-rutinoside (**11**), kaempferol-3-*O*-(4″/3″-acetyl)-neohesperidoside isomer (**12**).

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kaempferol-3-*O*-neohesperidoside (3,5,7,4′-tetrahydroxyflavone-3-*O*-rhamnosyl(1→2)glucoside) (**6**) (Table 1), besides other compounds at trace amounts. Table 1 shows the most frequent ions, which characterise the fragmentation of the flavonoid *O*-glycosides found. Other ions were observed, but they have not been included due to their low significance on the MS ions behaviour. The ions that are a consequence of the hexose internal cleavage (compounds **5**, **8** and **12**) are not included in Table 1, although they are discussed in the text and in Figs. 2–5. In addition to non-coloured compounds, petunidin-3-*O*-rutinoside, described before as the major anthocyanin,[1] was also noticed (retention time (*R*t): 12.6 min; UV: 275, 347sh, 529 nm; +MS: 625 $[M]^+$, $+MS^2[M]^+$: 317 (100%)).

The structure of compound **6** was established by means of its $-MS^2$ fragmentation, where ions corresponding to the losses of the rhamnosyl radical (−146 u) at *m*/*z* 447 and rhamnosyl plus water (−164 u) at *m*/*z* 429, besides the

Table 1. *R*t, UV, $[M–H]^-$ and $–MS^2[M–H]^-$ data of flavonoids from the hydro-alcoholic extract of bee pollen from *Echium plantagineum*[a]

					$-MS^2[M–H]^-$, *m*/*z* (%)					
Compounds[b]		*R*t (min)	UV (nm)	$[M–H]^-$	−42	−146	−162	−164[c]	−180[d]	[Aglc-H/2H]$^-$
1	Q-3Soph	11.7	256, 265sh, 353	625			463(10)		445(28)	300(100)
2	I-3Rut	12.5	—[e]	623						315(100)
3	Q-3Nhp	13.8	255, 265sh, 303sh, 354	609		463(5)		445(11)		300(100)
4	K-3Soph	16.0	—	609			447(7)		429(40)	285(100)
5	K-3(4Rh)Nhp	17.0	265, 293sh, 349	739		593(25)		575(100)		284(70)
6	K-3Nhp	17.9	265, 293sh, 347	593		447(10)		429(33)		284(100)
8	K-3(Act)Nhp	22.6	265, 295sh, 347	635	593(10)	489(6)		471(25)		284(100)
9	K-3Nhp-7Rh	24.1	—[e]	739		593(100)				285(8)
10	K-3Glc	25.6	265, 295sh, 346	447						285(100)
11	K-3Rut	26.0	265, 293sh, 347[f]	593						285(100)
12	K-3(Act)Nhp isomer	26.0	265, 293sh, 347[f]	635	593(6)	489(6)		471(10)		284(100)

[a] Main observed fragments. Other ions were found, but they have not been included.
[b] Q: quercetin (3,5,7,3′,4′-pentahydroxyflavone); I: isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone); K: kaempferol: (3,5,7,4′-tetrahydroxyflavone); Soph: sophoroside (glucosyl(1→2)glucoside); Rut: rutinoside (rhamnosyl(1→6)glucoside); Nhp: neohesperidoside (rhamnosyl(1→2) glucoside); Act: acetyl.
[c] −164: −(146+18).
[d] −180: −(162+18).
[e] Compounds hidden by others or in traces. Their UV spectra have not been properly observed.
[f] UV spectra of **11**+**12**.

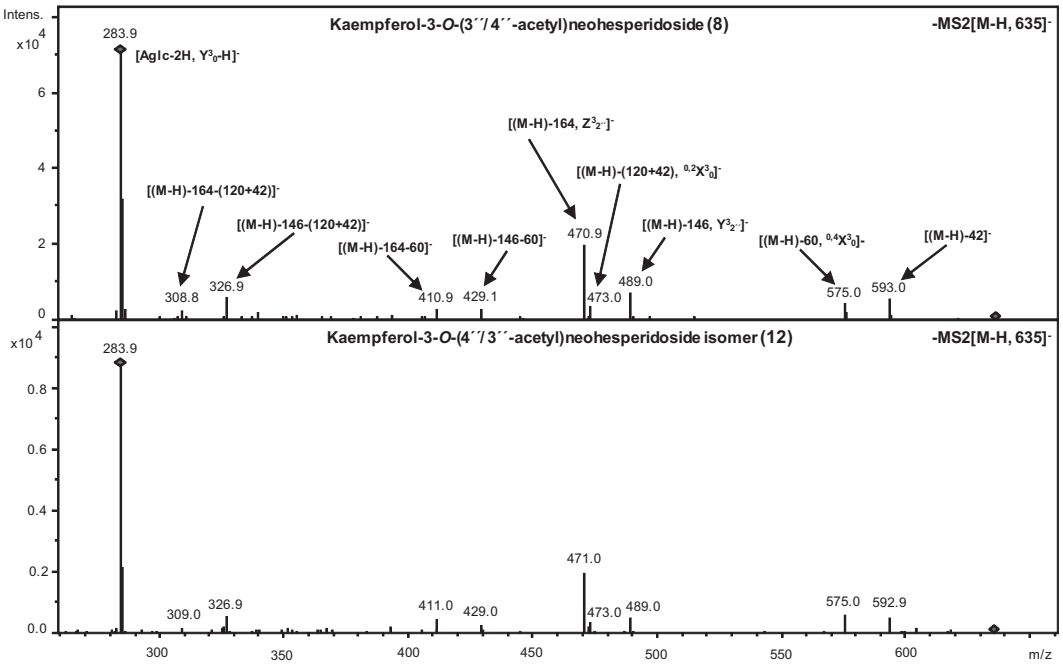

Figure 2. $–MS^2[M–H]^-$ mass spectra of kaempferol-3-*O*-(3″/4″-acetyl)neohesperidoside (**8**) and kaempferol-3-*O*-(4″/3″-acetyl)neohesperidoside isomer (**12**).

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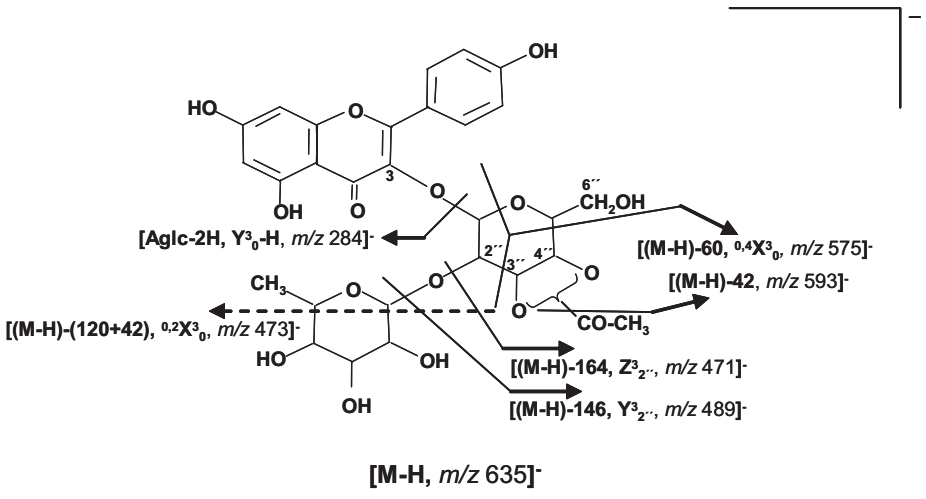

Figure 3. –MS^2[M–H]$^-$ fragmentation of kaempferol-3-*O*-(3″/4″-acetyl)neohesperidosides (**8** and **12**).

deprotonated ion of kaempferol as base peak (*m*/*z* 284, [kaempferol-2H]$^-$) were found. This fragmentation is characteristic of rhamnoglucosides with (1→2) interglycosidic linkage (neohesperidosides), when compared to rhamnosyl(1→6)glucosides (rutinosides).[14] Its UV spectra indicates that the position of the glycosylation in the hydroxyl group of the aglycone occurs in **3**.[15]

One isomer of this compound is compound **11** (kaempferol-3-*O*-rutinoside), whose –MS^2 fragmentation does not exhibit the ions that arise from the cleavage of the interglycosidic linkage (Table 1), which is characteristic of rhamnosyl(1→6) glucosides.[14] According to these same considerations, and differing solely in the aglycones, the structures of isorhamnetin-3-*O*-rutinoside (3,5,7,4′-tetrahydroxy-3′-methoxyflavone-3-*O*-rhamnosyl(1→6)glucoside) (**2**) and quercetin-3-*O*-neohesperidoside (3,5,7,3′,4′-pentahydroxyflavone-3-*O*-rhamnosyl(1→2)glucoside) (**3**) were established.

Compounds **1** and **4** are dihexosides of quercetin and kaempferol, respectively. In their –MS^2 fragmentation, the ions corresponding to the break of the interglycosidc linkage can be found (Table 1) and, as with the neohesperidosides, are characteristic of the (1→2) union,[13] probably glucosyl(1→2) glucoside (sophorosides): (**1**) quercetin-3-*O*-sophoroside and (**4**) kaempferol-3-*O*-sophoroside. In addition, kaempferol-3-*O*-glucoside (**10**) was also characterised. Regarding compound **7** (*R*t 19.4; UV: 266, 295sh, 347 nm; -MS: 705 [M–H]$^-$, -MS^2 (705): 593 ([(M–H)–112]$^-$, 60%), 284 ([kaempferol–2H]$^-$, 100%), -MS^3 (705→593): 447(8%), 429(40%), 284(100%)), its MS^2 spectrum shows, by loss of 112 u, an ion at *m*/*z* 593, whose fragmentation is similar to that of compound **6**, hence being a kaempferol-3-

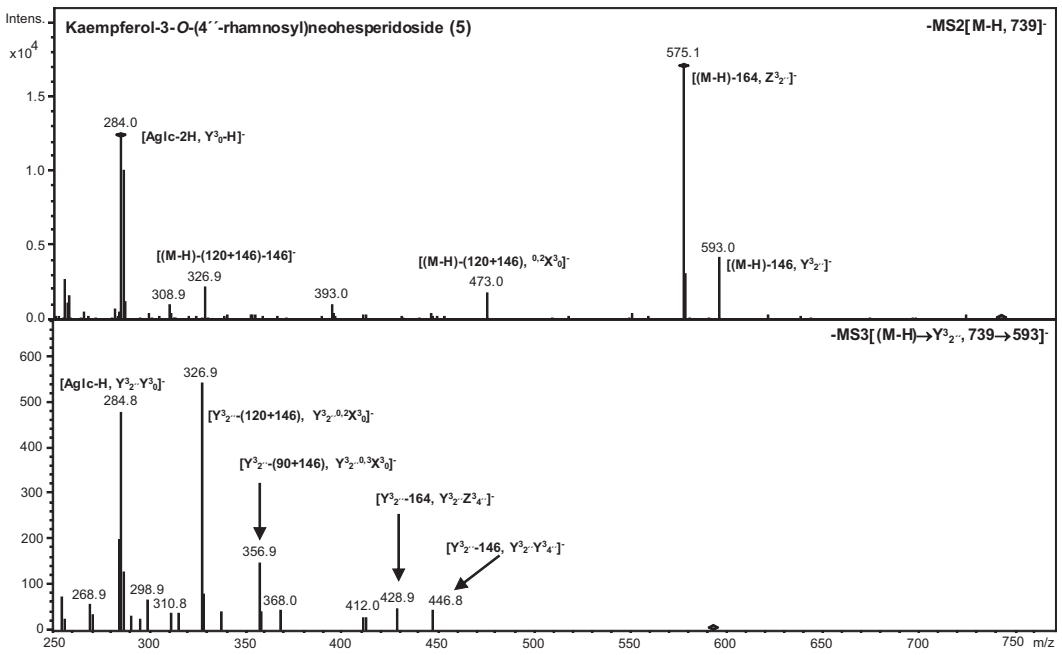

Figure 4. –MS^2[M-H]$^-$ mass spectra of kaempferol-3-*O*-(4″-rhamnosyl)neohesperidoside (**5**).

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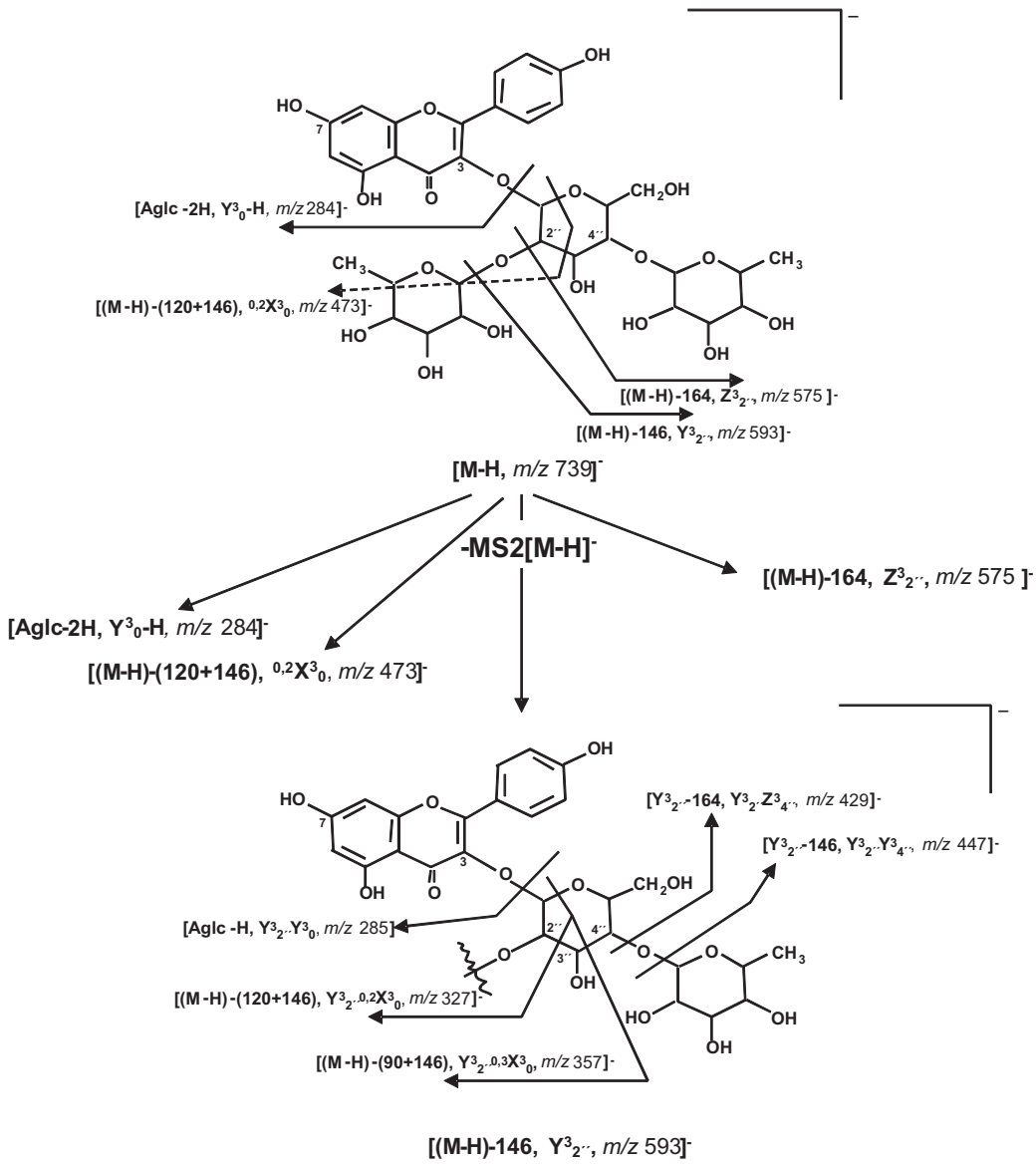

Figure 5. –MS^2[M–H]$^-$ and –MS^3[(M–H)→593]$^-$ fragmentation of kaempferol-3-*O*-(4″-rhamnosyl)neohesperidosides (**5**).

O-neohesperidoside derivative whose structure could not be totally characterised.

Other compounds that are derivatives of **6** are compounds **5** and **9** (rhamnosyl-kaempferol-3-*O*-neohesperidoside isomers) and compounds **8** and **12** (acetyl-kaempferol-3-*O*-neohesperidoside isomers). The acetylated derivatives (**8** and **12**) present a deprotonated molecular ion at *m*/*z* 635, which is consistent with a monoacetylation. The MS^2 fragmentation of both compounds is practically identical (Table 1, Figs. 2 and 3) and consists, besides the deprotonated ion of kaempferol as the base peak ([kaempferol–2H]$^-$), in the ion at *m*/*z* 593 resulting from the loss of the acetyl (−42 u) and the ions originated from the losses of rhamnosyl (−146 u) and rhamnosyl plus water (−164 u) at *m*/*z* 489 and 471, respectively, as expected from the rhamnosyl(1→2)glucoside interglycosidic linkage. These ions (489 and 471) still preserve the acetyl radical; thus, the losses of 146 and 164 u indicate that acetylation is not located in the rhamnose. The occurrence of other ions resulting from the internal cleavage of the hexose (not presented in Table 1) can provide some leads regarding the possible substitution. As so, the ion at *m*/*z* 473 (5%) ([(M–H)–(120+42), $^{0,2}X^3_0$]$^-$) produced from the loss of the glycosidic fraction that involves carbons 3″-6″, which still exhibits the acetyl moiety, indicates that the substitution must be located in the hydroxyls at 3″, 4″ or 6″ of the hexose (Figs. 2 and 3). The loss of the fragment of 60 u, that involves carbons 5″ and 6″ (Figs. 2 and 3) in which the acetyl radical (42 u) is already absent, resulting in the ion at *m*/*z* 575 (7%) ([(M-H)-60, $^{0,4}X^3_0$]$^-$), indicates that the acetylation is not at 6″. For this reason, the acetylation might be located in the hydroxyls at 3″/4″, with the position corresponding to each isomer remaining unknown. Other ions, less abundant, are those that are a consequence of simultaneous losses of fragment 60 and 146/164 at *m*/*z* 429 (3%) and 411 (4%), respectively, and (120+42) and 146/164 at *m*/*z* 327 (8%) and 309 (3%), respectively (Fig. 2).

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In relation to rhamnosyl-kaempferol-3-*O*-neohesperidoside isomers (**5** and **9**) the MS^2 fragmentation of **9** is characteristic of flavonol-3-*O*-glycosyl-7-*O*-glycosides,[13] showing as base peak the one that results from the loss of the glycosylation in 7 position (593, $[(M–H)–146]^-$), as well as the deprotonated ion of the aglycone in low abundance (Table 1). The $MS^3[(M–H)\rightarrow(M–H–146)]^-$ is identical to that of compound **6**, which indicates that it is kaempferol-3-*O*-neohesperidoside-7-*O*-rhamnoside.

For compound **5**, the MS^2 fragmentation shows a base peak at *m/z* 575, as a consequence of the loss of 164 u (rhamnosyl+water), besides the ion at *m/z* 593 (−146 u) and the deprotonated ion of kaempferol with a relative abundance of 70%. This fragmentation indicates that a single phenolic hydroxyl is glycosylated.[13] Another observed ion is that produced by the internal cleavage of the hexose with loss of the glycosidic fraction that involves carbons 3″-6″, to yield the ion at *m/z* 473 (10%) ($[(M–H)–(120+146), {}^{0,2}X^3_0]^-$) (Figs. 4 and 5, data not included in Table 1). This ion indicates that one of the rhamnoses is located in position 3″, 4″ or 6″, while the remaining one is at 2″, as expected. The ion resulting from the simultaneous loss of the fragments 120+146 and 146 (rhamnosyl in the 2″ position) was also detected at *m/z* 327. In the fragmentation of the ion $[Y^3_{2''}]^-$ ($-MS^3[(M–H)\rightarrow Y^3_{2''}$, $739\rightarrow593]^-$), besides the ions at *m/z* 285, 429 and 447 (due to the aglycone and to the losses of 164 u and 146 u, respectively), the ions at *m/z* 327 $[Y^3_{2''}–(120+146)$, $Y^3_{2''}{}^{0,2}X^3_0]^-$ and 357 $[Y^3_{2''}–(90+146)$, $Y^3_{2''}{}^{0,3}X^3_0]^-$ corresponding to the losses of the glycosidic fractions that involve the carbons 3″–6″ and 4″–6″, respectively, were noticed. These data indicate that the rhamnose that was still present in the $[Y^3_{2''}]^-$ ion is probably located in the 4″ or 6″ position. There are several facts that guarantee the substitution at 4″ more than at 6″: the abnormally high abundance of the ion $[(M-H)–164]^-$ (base peak) in the MS^2 fragmentation could be explained by the contribution of the losses of rhamnose+water in the 2″ and 4″ positions, which would not take place should the substitution be at 6″, given the fact that the interglycosidic linkage in 6″ is very stable, as was previously discussed concerning the different MS fragmentations of rutinosides and neohesperidosides.[14] In fact, the MS^2 of kaempferol-3-*O*-(2″,6″-dirhamnosyl)glucoside is different from that observed in compound **5**, which presents as base peak the deprotonated ion of kaempferol and the ion at *m/z* 575 with a relative abundance of 60%.[16] On the other hand, hindrance of the hydroxyl at 6″ would produce a delay in the elution in the reversed phase, with a subsequent raise in the *R*t with respect to that of kaempferol-3-*O*-neohesperidoside (compound **6**). For these reasons, this compound was tentatively identified as kaempferol-3-*O*-(4″-rhamnosyl)neohesperidoside.

CONCLUSIONS

In conclusion, the HPLC-PAD-MS^n analysis allowed characterisation for the first time of the non-coloured flavonoids on *E. plantagineum* bee pollen. The results obtained provide evidence of the effective value of HPLC-PAD-MS^n in the differentiation of flavonoids isomers, even when present in trace amounts, without the need for previous isolation and purification of the compounds. In addition to the demonstrated versatility of HPLC-PAD-MS^n, this work with bee pollen from *E. plantagineum* adds to the phytochemical knowledge of this species, thus being of great potential in quality control and detection of adulterations.

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