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Analysis of phenolic compounds from different morphological parts of *Helichrysum devium* by liquid chromatography with on-line UV and electrospray ionization mass spectrometric detection

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A simple and rapid method has been used for the screening and identification of the main phenolic compounds from *Helichrysum devium* using high-performance liquid chromatography with on-line UV and electrospray ionization mass spectrometric detection (LC-DAD/ESI-MS^n). The total aerial parts and different morphological parts of the plant, namely leaves, flowers and stems, were analyzed separately. A total of 34 compounds present in the methanolic extract from *Helichrysum devium* were identified or tentatively characterized based on their UV and mass spectra and retention times. Three of these compounds were positively identified by comparison with reference standards. The phenolic compounds included derivatives of quinic acid, *O*-glycosylated flavonoids, a caffeic acid derivative and a protocatechuic acid derivative. The characteristic loss of 206 Da from malonylcaffeoyl quinic acid was used to confirm the malonyl linkage to the caffeoyl group. This contribution presents one of the first reports on the analysis of phenolic compounds from *Helichrysum devium* using LC-DAD/ESI-MS^n and highlights the prominence of quinic acid derivatives as the main group of phenolic compounds present in these extracts. We also provide evidence that the methanolic extract from the flowers was significantly more complex when compared to that of other morphological parts.

Plants of the *genus Helichrysum* belong to the *Asteraceae* family, a name originating from the Greek words helios (sun) and chrysos (gold) that reflect the attractive yellow flowers displayed by several species of these genus.[1] This genus comprises more than 500 species mainly distributed in South Africa, although many endemic species can be found in southern Europe, south-west Asia, southern India, Sri Lanka and Australia. Several studies performed on *Helichrysum* species showed that they have a wide range of biological activities, such as antimicrobial, anti-inflammatory, anti-allergic, relief abdominal pain, heart burn, cough, cold and wounds.[2]

In Madeira Archipelago (Portugal) there are four endemic species together with several imported species of *Helichrysum* that are mostly used in horticulture and in folk medicine, especially in rural areas. *Helichrysum devium* Johns., the subject of this investigation, is one of those endemic subspecies that is used in folk medicine against respiratory diseases, such as bronchitis and pharyngitis. This plant faced near extinction due to massive collection of wild specimens. Fortunately, a successful programme of green house reproduction has facilitated its re-introduction in its natural habitat on the rocky slopes of the south-east cost of the island of Madeira.

The pharmacological activities of *Helichrysum* plants have been associated to several classes of compounds such as flavonoids, phloroglucinols, α-pyrones, coumarins and terpenoids which have been previously described.[3] Previous studies have reported the occurrence of quinic acid derivatives esterified with one to three residues of caffeic acid.[2,4] A few studies using analysis by liquid chromatography with diode-array detection coupled with mass spectrometric detection (LC-DAD/MS^n) also described the characterization of phenolic compounds from *Helichrysum* species. Carini *et al*.[2] studied *Helichrysum stoechas* and found the presence of some phenolic compounds, namely caffeoylquinic acid and flavonol derivatives, with potent antioxidant properties.

Phenolic compounds are a class of low molecular weight compounds which are secondary metabolites synthesized by the plants during normal development and in response to stress conditions like infection, wounding and UV radiation.[5] These compounds are not only associated with the colour, flavour and taste in many plants, but are also reported to have valuable medicinal properties such as protection against cancer, cardiovascular and neurodegenerative dis-

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eases.[6] For these reasons, many studies have been performed in order to identify and characterize phenolic compounds from natural sources.

The main classes of phenolic compounds are flavonoids and phenolic acids (e.g. hydroxybenzoic and hydroxycinnamic acids).

Flavonoid conjugates represent a very large and diverse group of phenolic compounds with similar structure having a common C6-C3-C6 flavone skeleton.[7] In cell plants, flavonoids may occur in modified forms corresponding to additional hydroxylation, methylation and/or glycosylation. It is also possible to have aromatic and aliphatic acids, sulfate, prenyl, methylenedioxyl or acyl groups also attached to the flavonoid skeleton or its glycoside moieties. Flavonoid glycosides are the most common phenolic compounds and are divided according to the site of the flavonoid aglycone where the sugar moiety is attached. *O*-Glycosides have glycoside groups connected to hydroxyl groups while in the *C*-glycosides the sugar bond connects the carbon atoms in ring A.

Since phenolic compounds are usually found as complex mixtures in plant extracts, efficient and selective analytical methods are required to analyze them. Liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) has proved to be a very powerful tool for the analysis of these compounds. According to several studies, using either APCI or ESI, the negative ionization mode typically provides enhanced sensitivity and yields complementary information. However, detection in the positive ion mode provides useful structural information for MS/MS characterization. A comparison between ESI and APCI indicated that the latter ionization mode is favoured for the analysis of phenolic compounds of plant extracts due to its higher ionization efficiency and selectivity for these compounds.[8–10] The mass spectra of flavonoids obtained with quadrupole and ion-trap instruments are typically very similar, even though differences in the relative abundances of fragment and adduct ions have been noted.[11]

The aim of this work was to characterize by LC-DAD/ESI-MS^n the main phenolic compounds present in the methanolic extracts of *Helichrysum devium*. Since the use of this plant in folk medicine shows variation in activity depending on the parts of the plant used (flowers only, leaves only or total aerial parts), it is important to evaluate the distribution of phenolic compounds in different morphological parts and correlate their contribution to the biological activity. As part of this study we used negative ion mode in LC/MS and LC/MS^n analysis. A total of 34 compounds were identified or tentatively characterized, including flavonoids and quinic acid derivatives. This work represents a first detailed analysis on the distribution and characterization of these bioactive compounds from the different parts of the plant.

EXPERIMENTAL

Chemical and materials

Standards used for identification purposes with LC/ESI-MS^n were as follows: apigenin-7-*O*-glucoside (>99%), apigenin (>99%), luteolin (>99%), quercetin (>99%) were purchased from ExtraSynthese and 5-*O*-caffeoylquinic acid (99%), kaempferol (>99%) from Acros Organics. Stock solutions of these compounds (100 μg/mL) were prepared in ethanol and further analysed by LC-DAD/ESI-MS^n.

HPLC grade acetonitrile (CH_3CN) (Lab-Scan, 99%) and ultra-pure water (Milli-Q, Waters) were used for all analysis. The methanol used for extraction of *Helichrysum devium* was AR grade, purchased from Fisher. Eluents prepared for LC/MS analysis (formic acid 0.1%, v/v) were additionally filtered through a 0.45 μm membrane (Millipore).

Sample preparation

Samples of *Helichrysum devium* were collected in the wild and identified by taxonomist Fátima Rocha and a voucher was deposited in the Madeira Botanical Garden Herbarium collection. Dried and powdered plant material (total aerial parts, 100 g) was exhaustively extracted by maceration with methanol (1 L), at room temperature for 24 h, yielding 8.64 g of dry extract.

For assessment of morphological parts, the leaves, flowers and stems of the plant were collected, dried and powdered separately. Each sample was extracted sequentially with four solvents of increasing polarity (*n*-hexane, chloroform, ethyl acetate and methanol), at room temperature for 24 h.

In all cases the solutions were filtered and concentrated to dryness under reduced pressure in a rotary evaporator (40°C). At this stage only methanolic extracts were used for the LC-DAD/ESI-MS^n analysis. Stock solutions with concentrations (w/v) of 5 mg/mL were prepared by dissolving dried extract in initial HPLC mobile phase (ACN/H_2O, 20:80).

These solutions were filtered through 0.45 μm micropore membranes prior to use and volumes of 10 μL were injected for LC-DAD/ESI-MS^n analysis. Three independent assays were performed for each sample.

LC conditions

HPLC analysis was performed on a Dionex ultimate 3000 series instrument coupled to a binary pump, a diode-array detector (DAD), an autosampler and a column compartment. The wavelength range was set at 210–520 nm and was monitored at 280 nm. Samples were separated on a Phenomenex Gemini C_{18} column (5 μm, 250 × 3.0 mm i.d., Phenomenex) with a sample injection volume of 10 μL. The mobile phase consisted of acetonitrile (A) and water/formic acid (100:0.1, v/v) (B). A gradient program was used as follows: 20% A (0 min), 25% A (10 min), 25% A (20 min), 50% A (40 min), 100% A (42–47 min), 20% A (49–55 min). The mobile phase flow rate was 0.4 mL/min; the chromatogram was recorded at 280 nm and spectral data for all peaks were accumulated in the range of 190–400 nm. Column temperature was controlled at 30°C.

Mass spectrometric conditions

For LC/ESI-MS analysis, a model 6000 ion trap mass spectrometer (Bruker Esquire, Bremen, Germany) fitted with an ESI source was used. Data acquisition and processing were performed using Esquire control software. Negative

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ion mass spectra of the column eluate were recorded in the range *m/z* 100–1000 at a scan speed of 13000 Da/s. High-purity nitrogen (N_2) was used both as drying gas at a flow of 10.0 mL/min and as a nebulizing gas at a pressure of 50 psi. The nebulizer temperature was set at 365°C and a potential of +4500 V was used on the capillary. Ultra-high-purity helium (He) was used as collision gas at a pressure of 1×10^{-5} mbar and the collision energy was set at 40 V.

The acquisition of MS^n data was made in *auto* MS^n mode, with isolation width of 4.0 *m/z*. For MS^n analysis, the mass spectrometer was scanned from 10 to 1000 *m/z* with a fragmentation amplitude of 1.0 V (MS^n up to MS^4) and two precursor ions.

RESULTS AND DISCUSSION

Three independent assays were performed for the analysis of the methanolic extracts from *Helichrysum devium* by LC-DAD/ESI-MS^n and no relevant variation were observed that can be related to the nature of detected fragments and their relative intensities.

The base peak chromatogram profiles of the four methanolic extracts under analysis are shown in Fig. 1. As can be seen, the majority of compounds could be well separated.

Whenever possible, the HPLC retention time, UV and mass spectra of detected compounds were compared with reference standards. Because only a few reference compounds were available, structures of unknown compounds were characterized based mainly on their own MS^n fragmentation behaviour, on retention times and on studies of their UV spectra.

The UV profile and spectral similarities were useful characteristics for the establishment of classes of detected compounds. The hydroxycinnamic acid derivatives showed two maximum absorption bands at 230–240 nm and 320–330 nm, together with a shoulder around 300–310 nm. Flavonols and their glycosides exhibited two maximum absorptions at 250–270 nm and 320–360 nm, derived from the aglycone A and B rings, respectively. Peaks corresponding to flavone glycosides showed three absorptions at 210–230 nm, 250–280 nm and 330–350 nm.

The structures were further and more fully characterized based on their MS^n fragmentation behaviour. MS^n fragmentation ions of the compounds detected in all extracts are presented in Tables 1–4 and their chemical structures are shown in Fig. 2.

An essential step in the LC-DAD/ESI-MS^n analysis was to determine the molecular weight of each detected compound. Most of the phenolic compounds gave deprotonated molecular ions $[M–H]^-$ of high abundance, which allowed them to undergo MS^n analysis. Usually, the most abundant peak in a full MS spectrum was assigned to $[M–H]^-$ and this assignment was more consistent if adduct ions and dimers were present.[12]

Among the identified compounds, there were hydroxycinnamic acids, flavonoids (flavonol and flavone type), caffeic acid and a protocatechuic acid derivative.

Identification will be presented in the next subsections, grouping the compounds by the nature of the respective aglycones. Compounds were numbered by their order of elution and this numeration was kept identical for all samples. Some of the compounds were present in all analyzed extracts while some were absent from one or more morphological parts.

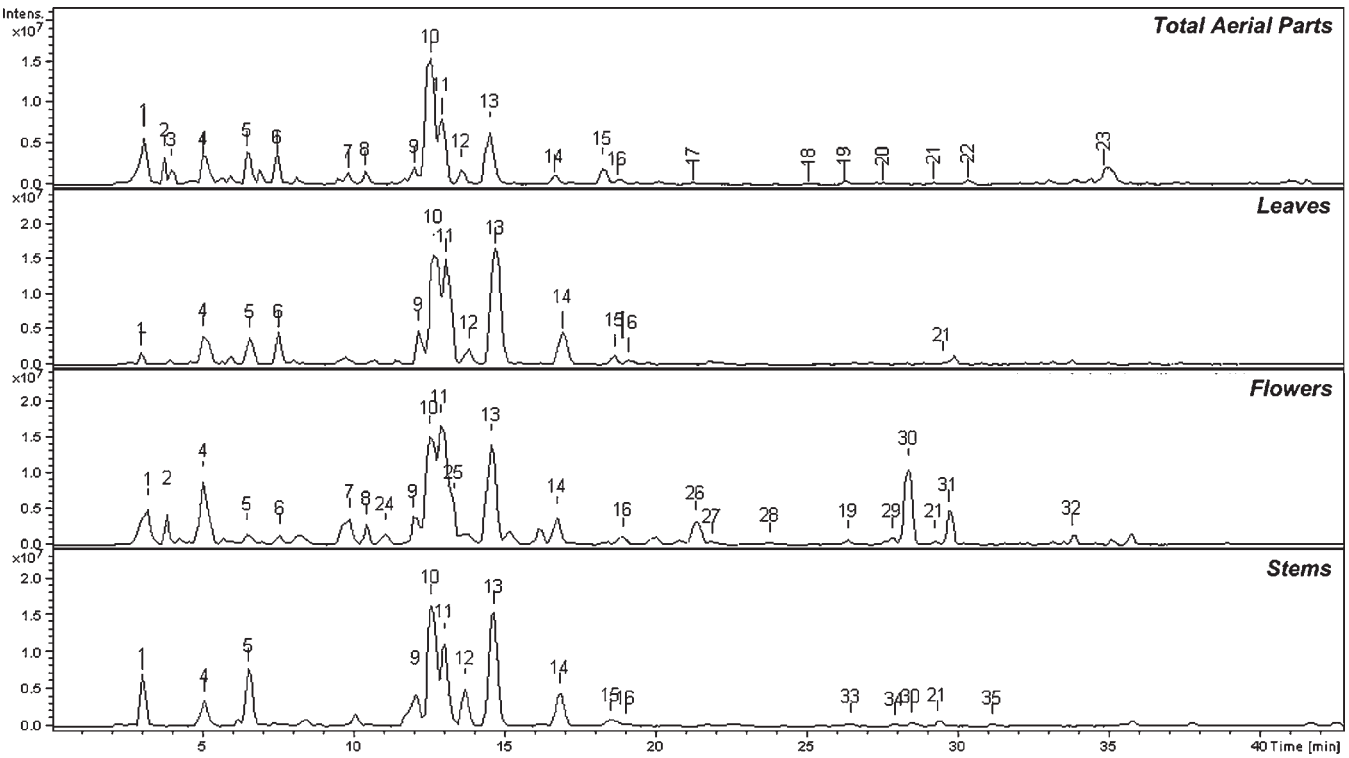

Figure 1. HPLC-DAD/ESI-MS^n analysis of the methanolic extracts of *H. devium* – LC/MS-negative ion ESI-MS base peak chromatogram (BPC).

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Table 1. Characterization of phenolic compounds of the methanolic extract of total aerial parts from *Helichrysum devium* by LC-DAD/ESI-MS^n

No.	t_R (min)	UV λ_{max} (nm)	$[M-H]^-$ *m/z*	LC-DAD/ESI-MS^n *m/z* (% base peak)	Identification
1[†]	3.1	266	191	MS^2 [191]: 173 (46.2), 127 (100), 111 (30.7) MS^3 [191→127]: 109 (100)	Quinic acid
2[a,c,d]	3.7	254, 287	317	MS^2 [317]: 225 (100), 165 (25.3), 125 (34.4) MS^3 [317 → 225]: 207 (56.4), 165 (100), 125 (81.9)	Unknown
3[a]	3.9	279	491	MS^2 [491]: 441 (15.4), 424 (11.1), 423 (100) MS^3 [491→423]: 395 (100), 263 (66.2), 173 (71.2) MS^4 [491→423→395]: 263 (76.2), 161 (100)	Unknown
4[*,†]	5.0	242, 300, 325	353	MS^2 [353]: 191 (100), 173 (3.7) MS^3 [353→191]: 173 (25.9), 127 (100), 111 (30) MS^4 [353→191→127]: 109 (100)	5-*O*-Caffeoylquinic acid (Chlorogenic acid)
5[†]	6.5	239, 303, 321	515	MS^2 [515]: 353 (100), 335 (34.6), 191 (19), 179 (41.5) MS^3 [515→353]: 191 (100), 179 (57.9) MS^4 [515→353→191]: 173 (15.6), 127 (100), 109 (67.6)	1,3-*O*-Dicaffeoylquinic acid
6[a,b,c]	7.5	228, 297, 329	429	MS^2 [429]:393 (100) MS^2 [393]: 191 (23.2), 149 (100), 131 (42.1) MS^3 [429→393]: 191 (31.6), 149 (100), 131 (26.5) MS^3 [393→149]: 131 (100), 113 (20.5) MS^4 [429→393→149]: 131 (100), 113 (20.5) MS^4 [393→149→131]: 113 (100)	Unknown
7[a,c]	9.8	256, 346, 350	463	MS^2 [463]: 301 (100), 300 (26.6), 151 (5.5) MS^3 [463→301]: 271 (15.9), 255 (16.7), 179 (66.8), 151 (100) MS^4 [463→301→151]: 107 (100)	Quercetin-*O*-hexoside
8[a,c]	10.3	254, 271, 342	477	MS^2 [477]: 316 (13.4), 315 (100), 301 (10.6), 300 (43.6) MS^3 [477→315]: 301 (13.9), 300 (100) MS^4 [477→315→300]: 272 (75.4), 255 (100)	Isorhamnetin-*O*- hexoside
9[†]	12.1	246, 305, 322	515	MS^2 [515]: 353 (100), 335 (9.8), 179 (22.9), 173 (36) MS^3 [515→353]: 179 (63.4), 173 (100), 135 (20.1) MS^4 [515→353→191]: 155 (83.7), 111 (100), 109 (40.5)	3,4-*O*-Dicaffeoylquinic acid
10[†]	12.6	243, 300, 329	515	MS^2 [515]: 353 (100), 335(5.3), 191 (45.5) MS^3 [515→353]: 191 (100) MS^4 [515→353→191]: 173 (51.4), 127 (100), 111 (18.9), 109 (27.3)	1,5-*O*-Dicaffeoylquinic acid
11[†]	13	241, 298, 326	515	MS^2 [515]: 353 (100), 191 (11.9) MS^3 [515→353]: 191 (100), 179 (46.5), 135 (11.9) MS^4 [515→353→191]: 173 (100), 127 (91.5), 109 (57.4)	3,5-*O*-Dicaffeoylquinic acid
12[a,b,d]	13.6	244, 300, 328	601	MS^2 [601]: 557 (23.4), 515 (86.3), 395 (100) MS^3 [601→395]: 335 (60.6), 233 (100), 179 (47.1), 173 (54.9) MS^4 [601→395→233]: 191 (10.8), 173 (100)	Malonyl-1,4-*O*-dicaffeoylquinic acid
13[†]	14.5	245, 300, 328	601	MS^2 [601]: 557 (48.8), 515 (96.1), 395 (100), 233 (38.0) MS^2 [515]: 353 (100), 179 (12.4), 173 (24.3) MS^3 [601→395]: 335 (5.3), 233 (100), 173 (27.1) MS^3 [515→353]: 191 (34.8), 179 (53.0), 173 (100) MS^4 [601→395→233]: 173 (100) MS^4 [515→353→173]: 111 (100), 109 (17.9)	Malonyl-3,4-*O*-dicaffeoylquinic acid
14[†]	16.7	245, 298, 326	601	MS^2 [601]: 557 (28.8), 515 (45.1), 395 (100), 233 (39.7) MS^3 [601→395]: 335 (1.7), 233 (100), 173 (23.2) MS^4 [601→395→233]: 173 (100)	Malonyl-4,5-*O*-dicaffeoylquinic acid
15[a,b,d]	18.2	—	625	MS^2 [625]: 474 (26.0), 473 (100), 341 (5.4), 293 (17.7), MS^3 [625→473]: 341 (100), 293 (41.6), 233 (25.6), 179 (12.4) MS^4 [625→473→341]: 326 (12.5), 239 (29.8), 179 (100)	Caffeic acid derivative
16[†]	18.8	243, 330	529	MS^2 [529]: 367 (100), 353 810.3), 191 (26.8) MS^3 [529→367]: 191 (100), 173 (3.5) MS^4 [529→367→191]: 173 (42.0), 127 (100), 111 (56.9)	1-Caffeoyl-5-Feruloyl-quinic acid
17[a,c]	21.3	—	609	MS^2 [609]: 464 (20.7), 463 (100), 301 (36.0) MS^3 [609→463]: 301 (100), 300 (8.2), 179 (7.1) MS^4 [609→463→301]: 271 (19.4), 179 (60.6), 151 (100)	Quercetin *O*-rhamnosylhexoside
18[a]	25.0	—	445	MS^2 [445]: 399 (6.1), 238 (18.1), 237 (100) MS^3 [445→237]: 153 (100), 138 (14.7)	Protocatechuic acid derivative
19[a,c]	26.3	—	625	MS^2 [625]: 463 (40.9), 445 (17.9), 301 (100) MS^3 [625→301]: 271 (14.8), 179 (88.7), 151 (100) MS^4 [625→301→151]: 107 (100)	Quercetin-*O*-dihexoside
20[a]	27.5	—	711	MS^2 [711]: 667 (100) MS^3 [711→667]: 625 (38.6), 505 (100), 487 (51.1), 301 (98.3) MS^4[711→667→505]: 463 (19.1), 301 (100), 300 (51.1)	Quercetin-7-*O*-hexoside-3-*O*-(malonyl)-hexoside
21[†]	29.2	—	677	MS^2 [677]: 515 (100), 353 (16.8)	3,4,5-*O*-Tricaffeoylquinic acid

(*Continues*)

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Table 1. (Continued)

No.	t_R (min)	UV λ_{max} (nm)	$[M–H]^-$ *m/z*	LC-DAD/ESI-MS^n *m/z* (% base peak)	Identification
				MS^3 [677→515]: 353 (100), 179 (35.1), 173 (49.3) MS^4 [677→515→353]: 191 (33.23), 179 (65.9), 173 (100)	
22[a]	30.3	—	629	MS^2 [629]: 475 (41.4), 463 (100) MS^3 [629→463]: 343 (18.20), 301 (54.2), 300 (100) MS^4 [629→463→300]: 272 (34.2), 271 (100), 254 (22.7), 151 (44.6)	Quercetin hexoside derivative
23[a]	34.8	—	331	MS^2 [331]: 155 (100), 140 (27.3), 125 (35.5) MS^3 [331→155]: 140 (100), 125 (9.1) MS^4 [331→155→140]: 125 (100)	Unknown

[*] Compared with standard compound.
[†] Detected in all extracts.
[a] Detected in total aerial parts.
[b] Detected in leaves.
[c] Detected in flowers.
[d] Detected in stems.

Identification of hydroxycinnamic acid derivatives

In this work several hydroxycinnamic acid derivatives were identified by LC-DAD/ESI-MS^n experiments and their chemical structures and identification are presented in Fig. 2 and Table 5, respectively.

The deprotonated molecular ion ($[M–H]^-$) was abundantly produced under the MS^n conditions for all hydroxycinnamic acid derivatives and the loss of the substitution groups is always referred to in respect to this ion.

Mono-, di- and tricaffeoylquinic acids (1, 4, 5, 9, 10, 11, 21)

Compound **1** occurred at a retention time of 3.1 min and exhibited a $[M–H]^-$ ion at *m/z* 191. Its MS^2 fragmentation produced a $[M–H–CO–2H_2O]^-$ ion at *m/z* 127 as base peak; a $[M–H–H_2O]^-$ ion at *m/z* 173 was also observed. Compound **1** was identified as quinic acid, taking into account its MS^n fragmentation pattern and literature data.[13]

It was reported previously[14] that the linkage position of acyl groups on quinic acid could be determined by the analysis of the $[M–H]^-$ ion MS^2 fragmentation. In general, the $[quinic\ acid–H]^-$ ion at *m/z* 191 appears as the base peak when the acyl group is linked to the 3-OH or 5-OH position; these two isomers can be further differentiated since the $[caffeic\ acid–H]^-$ ion at *m/z* 179 is more significant for 3-OH compounds. When the acyl group is connected to 4-OH, the $[quinic\ acid–H_2O–H]^-$ ion at *m/z* 173 will appear as the base peak.[14,15]

Identification of the detected quinic acid derivatives was performed based on these assumptions and by using the hierarchical key for the identification by LC/MS^n of caffeoylquinic and dicaffeoylquinic acids derivatives proposed by Clifford *et al*.[16]

Compound **4** ($t_R = 5.0$ min) was unequivocally identified as 5-*O*-caffeoylquinic acid (chlorogenic acid) by comparison of the retention time and mass spectra with those of a reference standard. This compound displayed a $[M–H]^-$ ion at *m/z* 353, and its MS^2 spectrum gave a $[quinic\ acid–H]^-$ ion at *m/z* 191 as the base peak and a $[caffeic\ acid–H]^-$ ion at *m/z* 179 (weak ion, ca. 3% of the base peak). The occurrence of 5-*O*-caffeoylquinic acid in plants of the *Helichrysum* genus is very common.[2]

In addition to the monocaffeoylquinic acid, several dicaffeoylquinic acid (diCQA) isomers and a tricaffeoylquinic acid (triCQA) were identified in *Helichrysum devium*.

Compounds **5**, **9**, **10** and **11** all gave molecular ions $[M–H]^-$ at *m/z* 515; their fragmentation in MS^2 spectra gave, as the base peak, a $[M–H–162]^-$ ion at *m/z* 353, indicating the presence of more than one caffeoyl group linked to different hydroxyl groups.

However, their MS^3 and MS^4 spectra of the *m/z* 353 ions were significantly different. The ion at *m/z* 191 was observed as the base peak for compound **5**, **10** and **11**, but the ion at *m/z* 173 was the base peak for compound **9** which, as mentioned above, indicates the presence of a 4-OH-substituted quinic acid.

According to the literature,[15] it is possible to distinguish the 3,4-diCQA from the 4,5-diCQA since the two isomers differ in the intensity of the MS^2 'dehydrated' ion at *m/z* 335 ($[M–H_2O–H^+]^-$). For 3,4-diCQA, the peak at *m/z* 335 is more intense (~15% of base peak). In contrast, for 4,5-diCQA this ion is barely detectable (<5% of base peak). The MS^2 spectrum of compound **9** exhibited a secondary ion at *m/z* 335 (~13% of base peak), thus compound **9** was plausibly identified as 3,4-*O*-dicaffeoylquinic acid.

It has been reported that 3,4-*O*-dicaffeoylquinic acid is more easily eluted from the reversed-phase column when compared with 3,5-*O*-dicaffeoylquinic acid. Based on this information and comparing its MS^n spectra and fragment intensities with the literature data,[15] compound **11** was identified as 3,5-*O*-dicaffeoylquinic acid.

Compounds **5** and **10** were identified as 1,3-*O*-dicaffeoylquinic and 1,5-*O*-dicaffeoylquinic, respectively. Their MS^3 spectra are quite different; compound **5** showed an ion at *m/z* 179 (~ 50% of the base peak), characteristic of a 3-OH-substituted quinic acid,[3] as discussed before, and which is absent in the spectrum of compound **10**. Moreover, as previously reported by Clifford *et al*.,[16] 1,3-diCQA eluted

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Table 2. Characterization of phenolic compounds of the methanolic extract of leaves from *Helichrysum devium* by LC-DAD/ESI-MS^n

No.	t_R (min)	UV λ_{max} (nm)	$[M–H]^-$ *m/z*	LC-DAD/ESI-MS^n *m/z* (% base peak)	Identification
1†	3.0	262, 310	191	MS^2 [191]: 173 (69.5), 127 (100) MS^3 [191→127]: 109 (100)	Quinic acid
4*,†	5.0	240, 300, 325	353	MS^2 [353]: 191 (100), 173 (3.2) MS^3 [353→191]: 173 (71.1), 127 (100), 111 (43) MS^4 [353→191→127]: 109 (100)	5-*O*-Caffeoylquinic acid
5†	6.5	242, 304, 320	515	MS^2 [515]: 353 (100), 335 (26.5), 191 (18.7), 179 (44.5) MS^3 [515→353]: 191 (100), 179 (48.9) MS^4 [515→353→191]: 173 (58.7), 127 (100), 109 (52.9)	1,3-*O*-Dicaffeoylquinic acid
6a,b,c	7.5	242, 295, 342	429	MS^2 [429]: 393 (100) MS^3 [429→393]: 149 (100), 131 (44.8), 113 (15.6) MS^4 [429→393→149]: 131 (100), 113 (15.3)	Unknown
9†	12.1	243, 293, 324	515	MS^2 [515]: 353 (100), 335 (13.9), 179 (34.4), 173 (36.1) MS^3 [515→353]: 191 (42.3), 179 (63.3), 173 (100) MS^4 [515→353→173]: 155 (13.3), 111 (100)	3,4-*O*-Dicaffeoylquinic acid
10†	12.6	243, 294, 327	515	MS^2 [515]: 353 (100), 335 (6.8), 191 (30.0), MS^3 [515→353]: 191 (100) MS^4 [515→353→191]: 173 (35.0), 127 (100), 111 (32.3), 109 (24.9)	1,5-*O*-Dicaffeoylquinic acid
11†	13	242, 302, 326	515	MS^2 [515]: 353 (100), 335 (6.8), 191 (9.3) MS^3 [515→353]: 191 (100), 179 (50.1), 135 (15.3) MS^4 [515→353→191]: 173 (74), 127 (100), 109 (82.9)	3,5-*O*-Dicaffeoylquinic acid
12a,b,d	13.6	244, 300, 326	601	MS^2 [601]: 557 (28.2), 515 (79.0), 395 (100) MS^3 [601→395]: 335 (77.1), 233 (100), 179 (73.1), 173 (54.9) MS^4 [601→395→233]: 173 (100)	Malonyl-1,4-*O*-dicaffeoylquinic acid
13†	14.7	244, 300, 328	601	MS^2 [601]: 557 (46.0), 515 (79.6), 395 (100); 233 (41.8); MS^3 [601→515]: 353 (100), 335 (5.7), 191 (42.3) MS^3 [601→395]: 335 (2.5), 233 (100), 173 (25.5) MS^4 [601→515→353]: 191 (24.1), 179 (62.9), 173 (100) MS^4 [601→395→233]: 173 (100)	Malonyl-3,4-*O*-dicaffeoylquinic acid
14†	16.9	244, 300, 328	601	MS^2 [601]: 557 (39.0), 515 (35.3), 395 (100), 233 (43.2) MS^3 [601→395]: 335 (2.5), 233 (100), 173 (24.5) MS^4 [601→395→233]: 173 (100)	Malonyl-4,5-*O*-dicaffeoylquinic acid
15a,b,d	18.6	243, 290, 328	625	MS2 [625]: 474 (19.9), 473 (100), 341 (5.8), 293 (13.8) MS3 [625→473]: 342 (22.4), 341 (100), 293 (49.7), 233 (35.7) MS4 [625→473→341]: 239 (46.4), 179 (100), 164 (21.9)	Caffeic acid derivative
16†	19.1	—	529	MS^2 [529]: 367 (100), 191 (23.8) MS^3 [529→367]: 191 (100) MS^4 [529→367→191]: 173 (12.1), 134 (100), 127 (16.0)	1-Caffeoyl-5-feruloylquinic acid
21†	29.2	300, 328	677	MS^2 [677]: 516 (21.0), 515 (100), 353 (15.0) MS^3 [677→515]: 353 (100), 191 (15.5), 179 (22.3), 173 (41.2) MS^4 [677→515→353]: 191 (45.3), 179 (54.5), 173 (100)	3,4,5-Tri-*O*-caffeoylquinic acid

*Compared with standard compound.
† Detected in all extracts.
[a] Detected in total aerial parts.
[b] Detected in leaves.
[c] Detected in flowers.
[d] Detected in stems.

much earlier than 1,5-diCQA and these two isomers can also be distinguished by the relative intensity of the MS^2 fragment at *m/z* 335 (1,3-diCQA, ~30%; 1,5-diCQA, ~7%, and 3,5-diCQA, not detectable).

Compound **21** appeared at a retention time (t_R) of 29.2 min and displayed a $[M–H]^-$ ion at *m/z* 677, easily losing a caffeoyl moiety (162 Da) to form a base peak ion at *m/z* 515 in the MS^2 spectrum. MS^3 and MS^4 spectra were identical with those described above for 3,4-diCQA (compound **9**). So, it can be inferred that compound **21** is either 1,3,4-triCQA or 3,4,5-triCQA. As, in general, CQAs with a larger number of free equatorial hydroxyl groups in the quinic acid residue are more hydrophilic than those with a larger number of free axial hydroxyl groups,[16] the long retention time of this compound suggests a hydrophobic compound. Thus, compound **21** was identified as 3,4,5-triCQA.

All compounds mentioned above were found in all analyzed methanolic extracts.

Malonylcaffeoylquinic acid (12, 13, 14)

Compounds containing a malonyl group usually show characteristic ions $[M–H–44]^-$ and $[M–H–86]^-$ in the ESI negative mode of fragmentation.[17]

Compounds **12** (t_R = 13.6 min), **13** (t_R = 14.5 min) and **14** (t_R = 16.7 min) showed a $[M–H]^-$ ion at *m/z* 601 and their MS^2 spectra gave ions $[M–H–44]^-$ at *m/z* 557 and $[M–H–86]^-$ at

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Table 3. Characterization of phenolic compounds of the methanolic extract of flowers from *Helichrysum devium* by LC-DAD/ESI-MS^n

No.	t_R (min)	UV λ_{max} (nm)	$[M-H]^-$ *m/z*	LC-DAD/ESI-MS^n *m/z* (% base peak)	Identification
1[†]	2.9	261	191	MS^2 [191]: 173 (74), 127 (100), 111 (33.3) MS^3 [191→127]: 109 (100)	Quinic acid
2[a,c]	3.7	232, 319	317	MS^2 [317]: 225 (100), 165 (33.0), 125 (17.1) MS^3 [317 → 225]: 207 (100), 125 (96.5), 165 (85.5)	Unknown
4[*,†]	5.0	241, 300, 325	353	MS^2 [353]: 191 (100), 173 (3.7) MS^3 [353→191]: 173 (25.9), 127 (100), 111 (30) MS^4 [353→191→127]: 109 (100)	5-*O*-Caffeoylquinic acid (Chlorogenic acid)
5[†]	6.5	244, 297, 321	515	MS^2 [515]: 353 (100), 335 (23.3), 191 (29.37), 179 (43.6) MS^3 [515→353]: 191 (100), 179 (48.0) MS^4 [515→353→191]: 173 (100), 127 (46.9)	1,3-*O*-Dicaffeoylquinic acid
6[a,b,c]	7.5	244, 280, 324	429	MS^2 [429]:393 (100) MS^2 [393]: 191 (18.3), 149 (100), 131 (35.9) MS^3 [429→393]: 251 (24.5), 191 (15.8), 149 (100), 131 (44.4) MS^3 [393→149]: 131 (100), 113 (52.1) MS^4 [429→393→149]: 131 (100), 119 (41.7) MS^4 [393→149→131]: 113 (100)	Unknown
7[a,c]	9.9	255, 352	463	MS^2 [463]: 301 (100), 300 (20.9), 151 (6.2) MS^3 [301]: 271 (19.6), 255 (18.6), 179 (100), 151 (84.4) MS^4 [179]: 151 (100), 107 (7.3)	Quercetin-*O*-hexoside
8[a,c]	10.4	255, 342	477	MS^2 [477]: 316 (11.6), 315 (100), 300 (42.0) MS^3 [477→315]: 301 (15.8), 300 (100) MS^4 [477→315→300]: 272 (52.9), 271 (59.8), 255 (51.6), 216 (100)	Isorhamnetin-*O*-hexoside
24[c]	11.0	209, 257, 343	461	MS2 [461]: 286 (17.6), 285 (100) MS3 [461→285]: 257 (5.8), 243 (70.5), 241 (52.7), 217 (93.1), 199 (99.9), 175 (100), 151 (28.1)	Luteolin-7-*O*-glucuronide
6[†]	12.1	243, 302, 327	515	MS^2 [515]: 353 (100), 335 (12.9), 179 (22.2), 173 (53.4) MS^3 [515→353]: 191 (22.9), 179 (65.9), 173 (100) MS^4 [515→353→173]: 155 (47.1), 111 (100), 109 (35.8)	3,4-*O*-Dicaffeoylquinic acid
7[†]	12.6	243, 300, 328	515	MS^2 [515]: 353 (100), 191 (54.6) MS^3 [515→353]: 191 (100) MS^4 [515→353→191]: 173 (25.1), 127 (100), 111 (37.1), 109 (16.4)	1,5-*O*-Dicaffeoylquinic acid
8[†]	13	242, 300, 328	515	MS^2 [515]: 353 (100), 191 (9.6) MS^3 [515→353]: 191 (100), 179 (46.2), 135 (15.6) MS^4 [515→353→191]: 173 (57.7), 127 (100), 111 (72)	3,5-*O*-Dicaffeoylquinic acid
25[*,c]	13.2	286, 332	431	MS^2 [431]: 270 (15.6), 269 (100) MS^3 [431→269]: 225 (83.9), 224 (55.8), 181 (62.3), 149 (29.5), 117 (100) MS^4 [431→269→224]: 197 (100), 195 (53.8)	Apigenin-7-*O*-glucoside
13[†]	14.5	244, 300, 327	601	MS^2 [601]: 557 (42.1), 515 (95.7), 395 (100), 233 (30.4) MS^3 [601→395]: 335 (5.2), 233 (100), 173 (19.7) MS^4 [601→395→233]: 173 (100)	Malonyl-3,4-di-*O*-caffeoylquinic acid
14[†]	16.9	244, 300, 328	601	MS^2 [601]: 557 (29.6), 515 (37.4), 395 (100), 233 (45.94) MS^3 [601→395]: 335 (3.4), 233 (100), 173 (33.2) MS^4 [601→395→233]: 173 (100)	Malonyl-4,5-di-*O*-caffeoylquinic acid
16[†]	18.8	234, 291, 328	529	MS^2 [529]: 367 (100), 353 (19.4), 191 (19.4), MS^3 [529→367]: 191 (100), 173 (6.2) MS^4 [529→367→191]: 173 (80.4), 127 (100)	1-Caffeoyl-5-feruloylquinic acid
17[a,c]	21.3	265, 314	609	MS^2 [609]: 464 (18.3), 463 (100), 301 (28.9) MS^3 [609→463]: 301 (100), 300 (30.0) MS^4 [609→463→301]: 271 (31.6), 255 (18.5), 179 (82), 151 (100)	Quercetin *O*-rhamnosylhexoside
26[c]	21.9	—	529	MS^2 [529]: 368 (13.0), 367 (100), 161 (13.0) MS^3 [529→367]: 191 (27.4), 179 (100), 161 (84.8), 135 (72.4) MS^4 [529→367→179]: 135 (100)	Caffeic acid derivative
27[c]	23.7	—	609	MS^2 [609]: 464 (17.9), 463 (100), 301 (28.6) MS^3 [609→463]: 301 (100), 300 (24.1) MS^4 [609→463→301]: 271 (24.2), 255 (15.4), 179 (100), 151 (69)	Quercetin *O*-coumaroylhexoside
19[a,c]	26.3	—	625	MS^2 [625]: 463 (41.3), 445 (22.8), 301 (100) MS^3 [625→301]: 273(14.7), 271 (17.7), 257 (8.4), 255 (12.3), 179 (100), 151 (95.6)	Quercetin dihexoside

(*Continues*)

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Table 3. (Continued)

No.	t_R (min)	UV λ_{max} (nm)	$[M-H]^-$ *m/z*	LC-DAD/ESI-MS^n *m/z* (% base peak)	Identification
				MS^4 [625→301→179]: 151 (100), 107 (16.3)	
28*,c	27.7	—	285	MS^2 [285]: 243 (46.4), 241 (83.9), 217 (26.5), 199 (72.1), 175 (100), 151 (29.2), 135 (4.2) MS^3 [285→175]: 147 (100)	Luteolin
29c,d	28.3	266, 313	593	MS^2 [593]: 447 (9.8), 307 (10.0), 285 (100) MS^3 [593→285]: 257 850.2), 255 (50.9), 229 (45.8), 167 (52.), 151 (100) MS^4 [151]: 107 (100)	Kaempferol-*O*-coumaroylhexoside
21†	29.2	—	677	MS^2 [677]: 515 (100), 353 (17.8) MS^3 [677→515]: 353 (100), 335 (13.5), 191 (15.6), 179 (25.0), 173 (32.1) MS^4 [677→515→353]: 191 (45.3), 179 (54.5), 173 (100)	3,4,5-*O*- Tricaffeoylquinic acid
30c	29.7	266, 311	593	MS^2 [593]: 447 (8.4), 307 (4.2), 285 (100) MS^3 [593→285]: 257 (52.5), 255 (22.3), 229 (13.6), 151 (100) MS^4 [151]: 107 (100)	Kaempferol-*O*-coumaroylhexoside
31*,c	33.7	267, 332	269	MS^2 [269]: 227 (25.4), 225 (100), 201 (39.9), 151 (42.5), 149 (74.1) MS^3 [269→225]: 198 (36.1), 183 (75.7), 181 (100)	Apigenin

*Compared with standard compound.
† Detected in all extracts.
[a] Detected in total aerial parts.
[b] Detected in leaves.
[c] Detected in flowers.
[d] Detected in stems.

m/z 515, indicating the presence of a malonyl residue in their structures.

For all compounds, the MS^2 fragmentation of the deprotonated molecular ion led to the formation of an ion at *m/z* 395 (base peak) due to the loss of 206 Da (acetyl – caffeoyl). Based on the occurrence of this fragment, it is possible to deduce that the malonyl group is attached to one caffeoyl group instead of being linked to the quinic acid structure. To our knowledge, this is the first time that this linkage is described for malonylcaffeoylquinic acid derivatives.

The base peak in all the MS^3 spectra was a $[M-H-44-162-162]^-$ ion at *m/z* 233 assigned to acetylquinic acid, as previously described by Zhang *et al*.[17] This acetylation can stabilize the ring structure of quinic acid, which was confirmed by the non-observation of ions corresponding to ring fragmentation.

The malonyl group should be attached to the caffeoyl group at the 3-OH position of the quinic acid structures. This evidence is supported by fragmentation of the ion at *m/z* 395, where a fragment at *m/z* 173 (~25%) is observed. This ion is due to the loss of a caffeoyl group linked to the 4-OH position.

For compound **13**, MS^2 fragmentation of the $[M-H-86]^-$ ion resulted in the identification of a 3,4-diCQA moiety, by comparison with the fragmentation of compound **9** (Fig. 3, Scheme 1).

As already mentioned, compounds **12** and **14** showed a similar fragmentation pattern when compared to compound **13**, but it was not possible to fragment the $[M-H-86]^-$ ion in order to establish the exact position where the caffeoyl moieties are attached. However, the occurrence of an ion at *m/z* 173 as the base peak in the MS^4 spectrum indicates the presence of a 4-OH linkage position in the quinic acid structure.

According to the rules for diCQA,[15] it was assumed that malonylcaffeoylquinic acid isomers have the same order of elution. So, accepting that 3,4-diCQA is more easily eluted from the reversed-phase column than 4,5-diCQA, compounds **12**, **13** and **14** were identified as malonyl-1,4-*O*-dicaffeoylquinic acid, malonyl-3,4-*O*-dicaffeoylquinic acid and malonyl-4,5-*O*-dicaffeoylquinic acid, respectively. These three compounds were detected in all extracts, with the exception of compound **12** which was not detected in the flowers extract.

Caffeoylferuloylquinic acid (16, 26)

It was possible to identify two feruloylquinic acid derivatives (compound **16** and **26**) in the four analyzed extracts. Compound **16** was detected for all extracts but compound **26** could only be detected in the flowers extract; both exhibited a $[M-H]^-$ ion at *m/z* 529.

MS^n fragmentation of compound **16** ($t_R = 18.9$ min) gave MS^2 and MS^3 base peaks at *m/z* 367 [feruloylquinic acid–H]$^-$ and *m/z* 191 [quinic acid–H]$^-$, respectively. Based on these fragments, this compound was characterized as a caffeoylferuoylquinic acid (CFQA) isomer.

Identification of compound **16** was tentatively made by referring to the hierarchical key developed by Clifford *et al*.[15] Since the MS^3 spectrum displayed an ion at *m/z* 191 as the base peak, this compound should be a 3-OH- or 5-OH-substituted quinic acid. If it was a 3-OH-substituted compound, the peak abundance at *m/z* 179 should be above 50% of the base peak, which it is not observed in this case, so compound **16** was plausibly identified as 1-*O*-caffeoyl-5-*O*-feruoylquinic acid.

Compound **26** ($t_R = 21.9$ min) yielded a different fragmentation behaviour when compared with compound **16**. The

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Table 4. Characterization of phenolic compounds of the methanolic extract of the stems from *Helichrysum devium* by LC-DAD/ESI-MS^n

No.	t_R (min)	UV λ_{max} (nm)	$[M–H]^-$ *m/z*	LC-DAD/ESI-MS^n *m/z* (% base peak)	Identification
1[†]	3.1	261	191	MS^2 [191]: 173 (100), 127 (74), 111 (52.5) MS^3 [191→173]: 125 (82.6), 109 (100)	Quinic acid
4[*,†]	5.0	243, 300, 325	353	MS^2 [353]: 191 (100), 179 (3.9) MS^3 [353→191]: 173 (61.8), 127 (78.7), 111 (100) MS^4 [353→191→111]: 109 (100)	5-*O*-Caffeoylquinic acid (Chlorogenic acid)
5[†]	6.5	243, 303, 321	515	MS^2 [515]: 353 (100), 335 (25.0), 191 (24.7), 179 (47.3) MS^3 [515→353]: 191 (100), 179 (41.8), 135 (10.8) MS^4 [515→353→191]: 173 (100), 127 (68.4), 111 (31.5)	1,3-*O*-Dicaffeoylquinic acid
9[†]	12.0	243, 301, 325	515	MS^2 [515]: 353 (100), 335 (9.8), 179 (26.6), 173 (44.8) MS^3 [515→353]: 191 (38.9), 179 (71.3), 173 (100), 135 (16.2) MS^4 [515→353→173]: 155 (47.1), 111 (100)	3,4-*O*-Dicaffeoylquinic acid
10[†]	12.6	243, 302, 327	515	MS^2 [515]: 353 (100), 335 (6.5), 191 (38.5) MS^3 [515→353]: 191 (100) MS^4 [515→353→191]: 173 (29.1), 127 (100), 109 (40.2)	1,5-*O*-Dicaffeoylquinic acid
11[†]	13	242, 298, 326	515	MS^2 [515]: 353 (100), 335 (1.3) MS^3 [515→353]: 191 (100), 179 (43.8) MS^4 [515→353→191]: 173 (100), 127 (55.2), 109 (28.1)	3,5-*O*-Dicaffeoylquinic acid
12[a,b,d]	13.6	244, 300, 329	601	MS^2 [601]: 557 (23.0), 515 (100), 395 (96.1) MS^3 [601→515]: 353 (100), 299 (22.6), 203 (42.2) MS^4 [601→515→353]: 191 (18), 179 (47.3), 173 (100)	Malonyl-1,4-*O*-dicaffeoylquinic acid
13[†]	14.5	245, 302, 328	601	MS^2 [601]: 557 (52.8), 515 (74.1), 395 (100), 233 (34.2) MS^3 [601→395]: 335 (3.6), 233 (100), 173 (19.8) MS^4 [601→395→233]: 173 (100)	Malonyl-3,4-*O*-dicaffeoylquinic acid
14[†]	16.9	244, 300, 328	601	MS^2 [601]: 557 (27.9), 515 (37.2), 395 (100), 233 (43.2) MS^3 [601→395]: 335 (2.6), 233 (100), 173 (27.1) MS^4 [601→395→233]: 173 (100)	Malonyl-4,5-*O*-dicaffeoylquinic acid
15[a,b,d]	18.4	261, 352	625	MS^2 [625]: 474 (17.1), 473 (100), 341 (8.7), 293 (13.0) MS^3 [625→473]: 341 (100), 293 (56.7), 233 (34.3), 179 (24.1) MS^4 [625→473→341]: 239 (287.99.8), 179 (100), 164 (32)	Caffeic acid derivative
16[†]	19.0	—	529	MS^2 [529]: 368 (12.4), 367 (100), 353 (13.2), 191 (18.2) MS^3 [529→367]: 193 (14.6), 191 (100) MS^4 [529→367→191]: 173 (12.0), 134 (100), 127 (36.3), 111 (22.8)	1-Caffeoyl-5-feruloylquinic acid
32[d]	26.4	—	583	MS^2 [583]: 422 (24.9), 421 (100), 335 (8.8), 259 (69.2), MS^3 [583→421]: 259 (100), 173 (29.7) MS^4 [583→421→259]: 173 (100), 155 (9.9)	Unknown
33[d]	27.9	—	583	MS^2 [583]: 422 (23.2), 421 (100), 299 (26.9), 255 (19.4) MS^3 [583→421]: 353 (100), 335 (74.5), 259 (72.5), 179 (43.2), 173 (53.6) MS^4 [583→421→353]: 179 (100), 173 (95.7), 135 (50.0)	Caffeic acid derivative
29[c,d]	28.3	—	593	MS^2 [593]: 447 (8.4), 307 (4.0), 285 (100) MS^3 [593→285]: 257 (68.9), 255 (24.6), 151 (100) MS^4 [151]: 107 (100)	Kaempferol-*O*-coumaroylglucoside
21[†]	29.2	242, 300, 325	677	MS^2 [677]: 515 (100), 353 (18.2) MS^3 [677→515]: 353 (100), 179 (21.0), 173 (37.6) MS^4 [677→515→353]: 191 (38.4), 179 (63.8), 173 (100)	3,4,5-*O*-Tricaffeoylquinic acid
34[d]	31.1	—	567	MS^2 [567]: 323 (100), 179 (28.1), 161 (19.9) MS^3 [567→323]: 179 (93.3), 161 (100), 135 (36.0) MS^4 [567→323→161]: 133 (100)	Unknown

[*]Compared with standard compound.
[†] Detected in all extracts.
[a] Detected in total aerial parts.
[b] Detected in leaves.
[c] Detected in flowers.
[d] Detected in stems.

MS^2 spectrum gave an ion at *m/z* 367, indicating the presence of a feruoylquinic acid residue but, in the further MS^n experiments, the presence of ferulic acid could not be confirmed. In the MS^3 spectrum, the base peak is the $[caffeoyl–H]^-$ ion at *m/z* 179. For these reasons, and with no further information, compound **26** could only be characterized as a caffeic acid derivative.

Identification of flavonoids compounds (7, 8, 17, 19, 20, 22, 24, 25, 27, 28, 29, and 30)

The present work led to the identification and characterization of a number of flavonoids with aglycones belonging to two subtypes: flavonols (quercetin, isorhamnetin and kaempferol) and flavones (luteolin and apigenin) (Fig. 2). Nearly all flavonoids were identified as glycosides contain-

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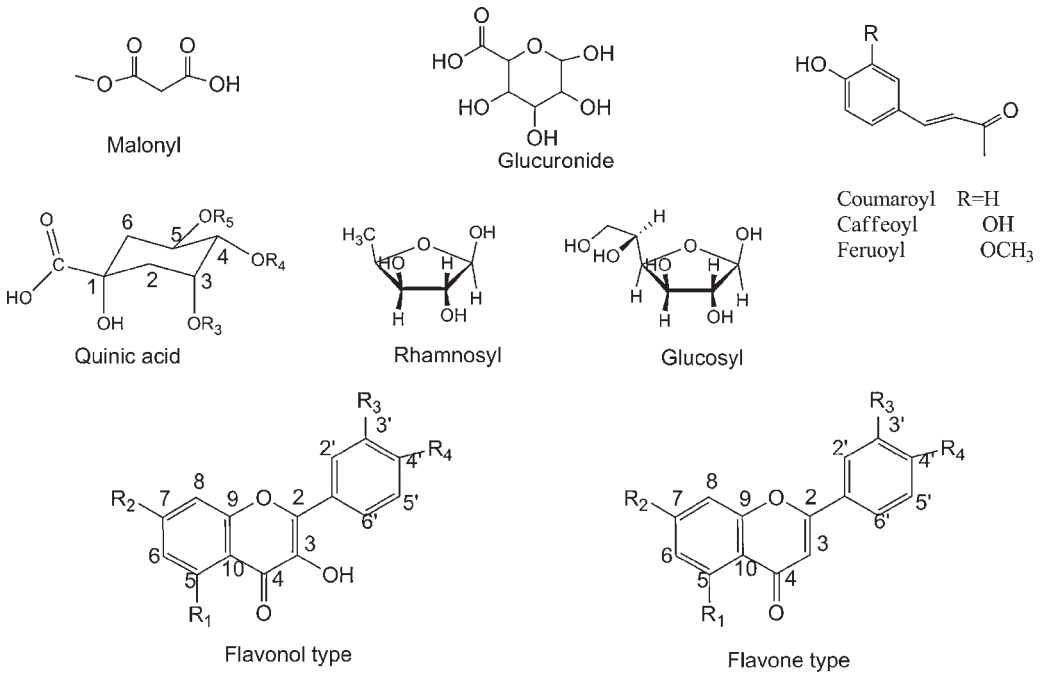


Flavonol type	R_1	R_2	R_3	R_4
Quercetin	OH	OH	OH	OH
Kaempferol	OH	OH	H	OH
Isorhamnetin	OH	OH	OCH_3	OH

Flavone type	R_1	R_2	R_3	R_4
Quercetin	OH	OH	OH	OH
Kaempferol	OH	OH	H	OH

Figure 2. Chemical structures of flavonoid aglycones and substitution groups identified in methanolic extracts from *Helichrysum devium*.

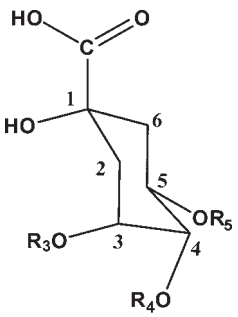
ing one or more sugar moieties and some were esterified with acyl groups. The MS^n fragmentation of these phenolic compounds showed the deprotonated molecular ion ($[M–H]^-$) and the deprotonated aglycone ion (Y_0^-) as a result of the loss of the sugar residue. The presence of hexoside, rhamnose, malonyl and glucunoride moieties was characterized by neutral losses of 162, 146, 146 and 176 Da, respectively. The flavonoid fragment ions were designated according to the nomenclature proposed by Ma *et al.*[18] (Fig. 4). For free aglycones, the $^{i,j}A^-$ and $^{i,j}B^-$ labels correspond to ions containing intact A- and B-rings, respectively, in which i and j indicate the C-ring bonds that have been broken (Fig. 4). For conjugated aglycones, Y_0^- is used to refer to the aglycone fragment [M–H–glycoside]$^-$.

Most of the identified flavonoids were exclusively detected in the flowers extract (**7**, **8, 17**, **19**, **24**, **25, 27**, **28, 29**, and **30**); compounds **7**, **8**, **17** and **19** were also detected in the total plant extract and compound **29** in the stems. Compounds **20** and **22** were only detected in the total plant extract.

Compound **7** ($t_R = 9.8$ min) yielded a $[M–H]^-$ ion at *m/z* 463 and its analysis by MS^2 resulted in the aglycone fragment (Y_0^-) at *m/z* 301 (loss of 162 Da, probably due to an hexoside residue). The MS^n fragmentation gave ions at *m/z* 151 ($^{1,2}A^-$–CO), 179 ($[^{1,2}A^-–H]^-$), 255 ($[M–H–H_2O–CO]^-$) and

Table 5. Quinic acid derivatives identified by LC/ESI-MS^n in different morphological parts of *Helichrysum devium*

Compound	R_1	R_3	R_4	R_5
1	H	H	H	H
4	H	H	H	Caffeoyl
5	Caffeoyl	Caffeoyl	H	H
9	H	Caffeoyl	Caffeoyl	H
10	Caffeoyl	H	H	Caffeoyl
11	H	Caffeoyl	H	Caffeoyl
21	H	Caffeoyl	Caffeoyl	Caffeoyl
12	Malonyl-Caffeoyl	H	Caffeoyl	H
13	H	Malonyl-Caffeoyl	Caffeoyl	H
14	H	H	Caffeoyl	Malonyl-Caffeoyl
16	Caffeoyl	H	H	Feruoyl



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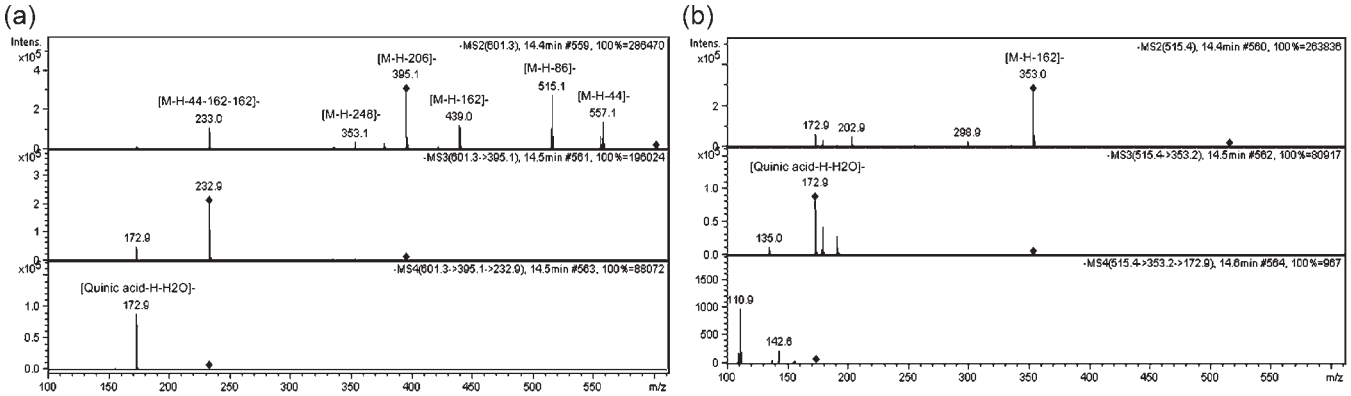


Figure 3. ESI-MS^n negative mode of compound **13**. Sequential fragmentation, MS^n (n = 2–4), of (a) the ion at *m/z* 601 and (b) the ion at *m/z* 515.

271 ([M–H–CH_2O]$^-$), originating from a retro-Diels-Alder (RDA) reaction.[19–21] Comparing these MS^n data with the fragmentation of a standard quercetin solution (data not showed) it is possible to observe that they are very similar and so quercetin should be the aglycone of compound **7**.

It is known that, despite the fact that any of the hydroxyl groups of the flavonoid aglycone can be glycosylated, certain positions are favoured. For flavonols the 3-OH and 7-OH positions are regular glycosylation sites.[10] Even so, based only on MS^n data, neither the nature of the hexoside residue nor the sugar linkage position to the aglycone could be determined. Thus, compound **7** was preliminary characterized as a quercetin-*O*-hexose.

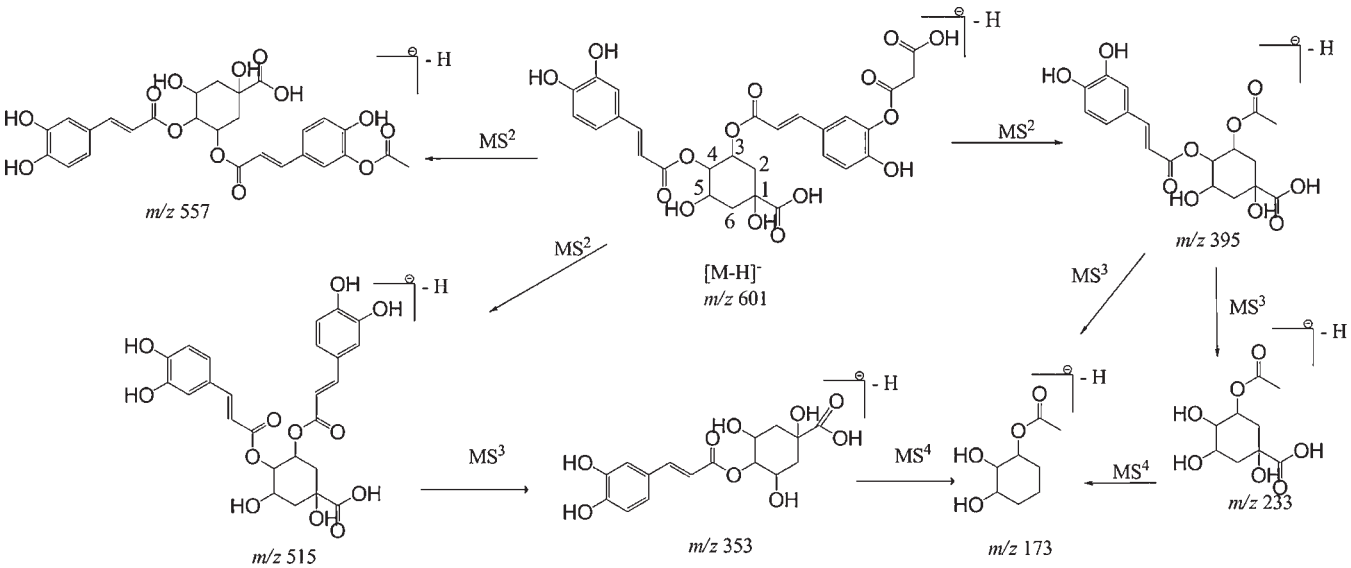
Compound **8** (t_R = 10.3 min) gave a molecular ion [M–H]$^-$ at *m/z* 477 and its MS^2 spectrum showed a fragment ion Y_{0^-} at *m/z* 315 (loss of 162 Da), suggesting the presence of a hexoside residue. Fragmentation of the ion at *m/z* 315 was very similar to that of isorhamnetin reported in previously studies.[14,22] So, compound **8** was tentatively identified as a isorhamnetin-*O*-hexoside.

Compounds **17** (t_R = 21.3 min) and **27** (t_R = 23.7 min) showed a very similar behaviour in the MS^n experiments and gave deprotonated molecular ions [M–H]$^-$ at *m/z* 609 and their MS^2 spectra showed a fragment ion [M–H–146]$^-$ at *m/z* 463 as base peak. The formation of the ion at *m/z* 463 can be assigned either to a loss of a rhamnose moiety or a coumaroyl group.

For both compounds the MS^3 spectra gave a base peak ion at *m/z* 301, corresponding to the deprotaned aglycone (Y_{0^-}), due to the loss of an hexoside residue: the corresponding aglycone radical ion [Y_0^-–H]$^-$ at *m/z* 300 (< 30% of the base peak) was also observed.

The fragmentation of the ion at *m/z* 301 produced ions at *m/z* 151 ($^{1,2}A^-$–CO), 179 ([$^{1,2}A^-$–H]) and 271 ([M–H–CH_2O]$^-$), leading to the aglycone identification as quercetin.

Flavonols substituted at 3-OH position should present relative high intensity aglycone radical fragment sometimes higher than the Y_0^- ion.[23] Such a pattern was not observed for compounds **17** and **27**; thus the glycosylation site cannot be surely confirmed. As mentioned above, either a malonyl or a rhamnosyl group could be attached to the hexoside residue but, based only on the MS^n data, it is hard to clearly make the attribution of either to compound **17** or **27**. However, it has been reported that, generally, flavonoid glycosides esterified



Scheme 1. Proposed fragmentation pathway for compound **13**.

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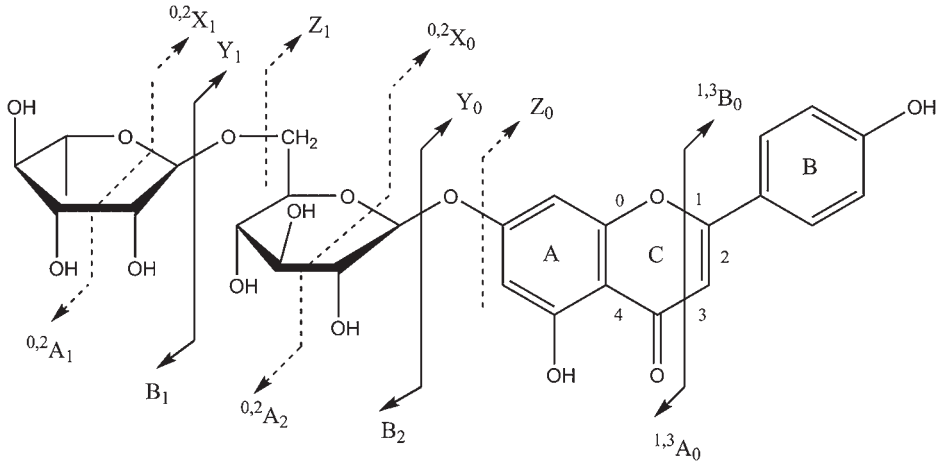

Figure 4. Ion nomenclature used for avonoid glycosides (illustrated on apigenin 7-*O*-rutinoside).[18]

with aromatic acids have higher retention times on RP-HPLC columns than diglycosides, monoglycosides and aglycones.[14]

With no further information, compounds **17** and **27** were tentatively identified as a quercetin *O*-rhamnosylhexoside derivative and quercetin *O*-coumaroylhexoside, respectively.

Compound **19** ($t_R = 26.3$ min) exhibited a $[M–H]^-$ ion at *m/z* 625. The MS^2 spectrum of this ion showed a fragment at *m/z* 301, corresponding to the loss of 324 Da, which indicates two hexoside moieties linked at the same position of the aglycone.[24] Fragmentation of the resulting aglycone ion, Y_0^-, produced characteristic fragments of quercetin. This compound was thus classified as a quercetin-*O*-dihexoside.

Compound **20** ($t_R = 27.5$ min) gave a $[M–H]^-$ ion at *m/z* 711. The fragmentation showed a loss of 44 Da, which indicates a decarboxylation from a dicarboxylic acid linked to the flavonoid glycoside. The MS^3 spectrum showed a base peak ion at *m/z* 505 (Y_0^{7-}) originating from the loss of a hexoside moiety (162 Da) and also a very intense peak at *m/z* 301 (Y_0^{3-}) (Fig. 5).

This type of fragmentation (Scheme 2), in which the loss of a sugar unit gives the most abundant base peak different from the base peak of the aglycone, indicates that there is a glycosylation in more than one phenolic hydroxyl group of the aglycone.[25] The fragmentation of the ion at *m/z* 505 yielded the aglycone fragment ion at *m/z* 301, by the loss of 204 Da from the decarboxylated malonyl group linked to the

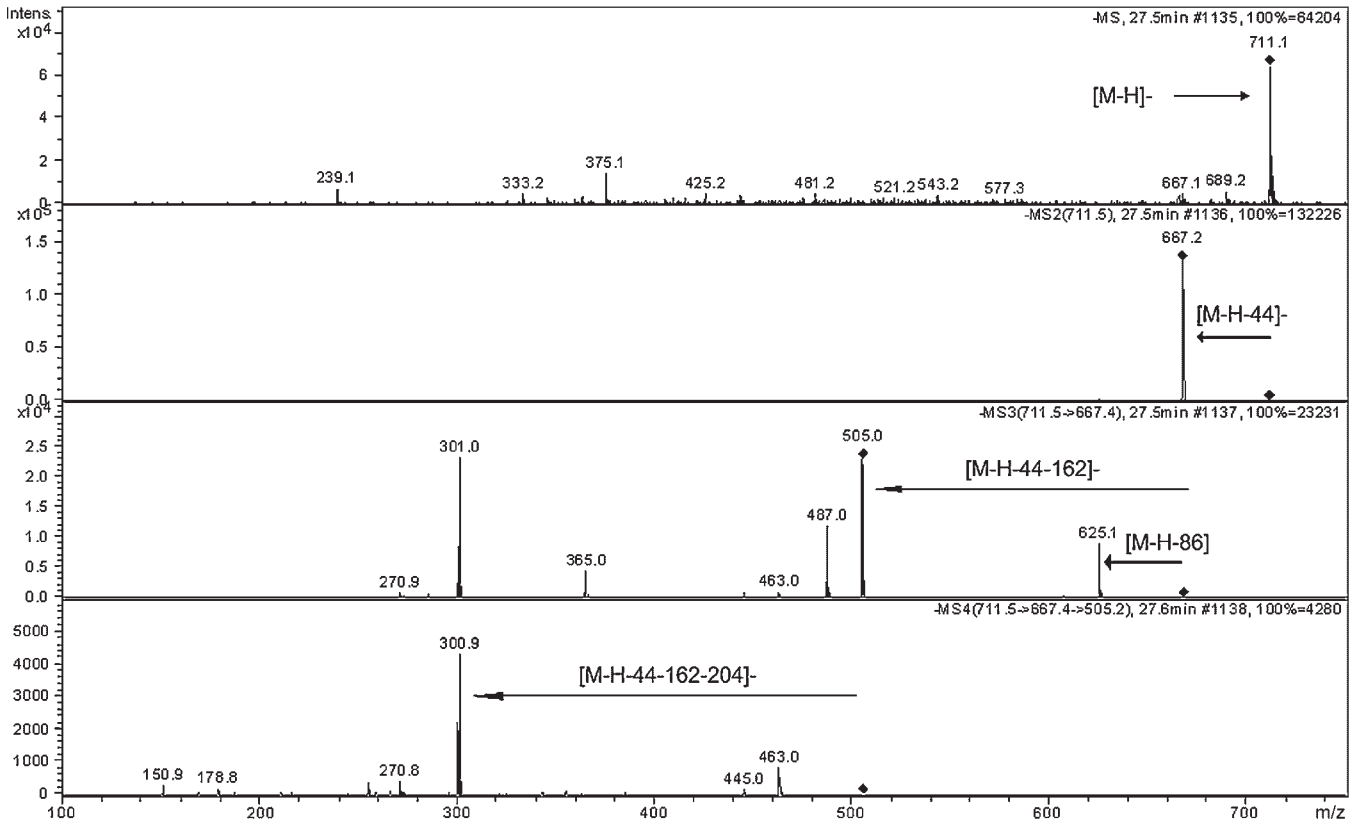

Figure 5. ESI-MS^n negative mode of compound **20**. Sequential fragmentation, MS^n (n = 2–4), of the ion at *m/z* 711.

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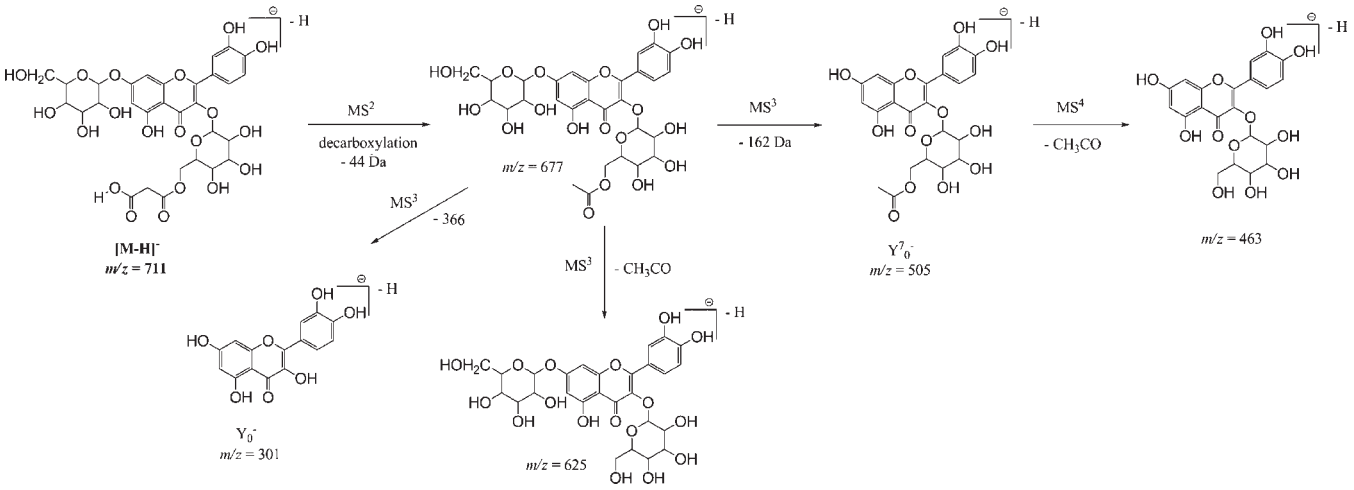

Scheme 2. Proposed fragmentation pathway for compound **20**.

hexoside residue. The aglycone was identified by MS^n fragmentation of the ion at *m/z* 301 as being quercetin.

The glycosylation sites were established attending to the guidelines presented by Ablajan *et al*.[9] In the MS^4 spectrum, the intensity of the fragment Y_0^- is higher than that of fragment $[Y_0^-–H]^-$, which implies a cleavage of an hexoside group at the 3-OH position. Therefore, the first sugar–aglycone bond to cleave is at the 7-OH position.

The exact location of the malonyl group on the hexoside part is difficult to define on the basis of obtained MS^n data, but it appears to be predominantly located at the 6-position of a hexoside moiety.[10] According to these MS^n data, compound **20** was plausible identified as quercetin-7-*O*-hexoside-3-*O*-(malonyl)hexoside.

Compound **22** ($t_R = 30.3$ min) exhibited a $[M–H]^-$ ion at *m/z* 629 and was identified as being a quercetin-*O*-hexoside derivative based on the MS^n fragmentation. The MS^2 spectrum showed a base peak ion at *m/z* 463, which corresponds to the loss of 166 Da (this fragment could not be identified based in the available data). The fragmentation of the ion at *m/z* 463 led to the formation of the same fragment ions detected for compound **7**.

Compounds **29** ($t_R = 28.3$ min) and **30** ($t_R = 29.7$ min) exhibited a very similar MS^n pattern and gave a molecular ion $[M–H]^-$ at *m/z* 593. Their MS^2 spectra contained a base peak ion $[M–H–146–162]^-$ at *m/z* 285 and a $[M–H–146]^-$ ion at *m/z* 447 (~10% of the base peak). As already known,[26] the neutral loss of 146 Da is characteristic of a coumaroyl group which was confirmed by the formation of a $[\text{coumaroylhexose–H}]^-$ ion at *m/z* 307. According to these considerations compounds **29** and **30** were preliminarily characterized as acylated flavonoid glycosides.

The peak at *m/z* 285 corresponds to the aglycone (Y_{0^-}–H) and its MS^n spectra showed a (Y_0^-–H–CO) ion at *m/z* 257, a (Y_{0^-}–2CO) ion at *m/z* 229 and, as base peak, an ion at *m/z* 151 ($^{1,3}A^-$), produced from a RDA reaction.[14] These RDA fragments are consistent with those found for a standard solution of kaempferol (MS^n fragmentation data not shown).

Theoretically, any of the kaempferol hydroxyl groups can be glycosylated, although certain positions are favoured: the 3-OH and 7-OH are the most common glycosylated positions.

As stated before, for flavonols glycosylated at the 3-OH position, the relative abundance of radical aglycone ion ($[Y_0^–H]^{-\cdot}$) is very pronounced.[23] However, this radical fragment was detected for both compounds but with a very low relative intensity (~4% of the base peak). So, glycosylation at the 3-OH position is not evident, leaving the 7-OH and 4′-OH positions as the most probable sites of glycosylation for these compounds. The 5-OH position is also available but 5-*O*-glycosides are very rare for compounds with a carbonyl at position 4, since the 5-OH group participates in hydrogen bonds with the adjacent 4-carbonyl group.[10]

As already mentioned, compounds **29** and **30** have an acyl group in their structures, but the exact location of the acyl group on the hexoside moiety is difficult to define based only on MS^n data. Acyl groups are predominantly located at the 6-position of a hexoside moiety,[21] but only when a $^{0,4}X$ fragment is present in the spectrum can the location at the 6-position be confirmed, which did not happen in this particular case.

With no further information, it was assumed that compounds **29** and **30** are kaempferol 7-*O*-coumaroylhexoside and kaempferol 4′-*O*-coumaroylhexoside.

Compound **28** ($t_R = 27.7$ min) gave a $[M–H]^-$ ion at *m/z* 285 and a $[2M–H]^-$ ion at *m/z* 571 (15.4% of base peak). The fragmentation of the molecular ion gave rise to several fragment ions at *m/z* 243 ($[M–H–C_2H_2O]^-$), 241 ($[M–H–CO_2]^-$), 217 ($[M–H–C_3O_2]^-$), 175 ($[M–H–C_3O_2–C_2H_2O]^-$), 199, 151 ($^{1,3}A^-$) and 135 ($^{1,3}B^-$). This compound was identified as luteolin by comparison of its MS^n fragmentation pattern with that of a reference standard (data not shown) and literature data.[19]

Compound **24** ($t_R = 11.0$ min) exhibited a $[M–H]^-$ ion at *m/z* 461. When submitted to further fragmentation this ion readily eliminated a glucuronic acid residue (observed by the loss of 176 Da) to produce the deprotonated aglycone ion Y_{0^-} at *m/z* 285. The glucuronic acid residue was confirmed by the MS^2 ions at *m/z* 357 and 327. The MS^3 spectrum of the aglycone ion gave fragments at *m/z* 243, 217, 199 and 175, characteristic ions of luteolin as described above. The favoured substitution position for flavones, like luteolin, is

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the 7-OH position.[10] Therefore, compound **24** was characterized as luteolin-7-*O*-glucuronide.

With a retention time of 13.2 min, compound **25** originated a molecular ion $[M–H]^-$ at *m/z* 431 and subsequent fragmentation showed the loss of 162 Da. The formed ion corresponds to a deprotonated aglycone ion at *m/z* 269. Prasain *et al*.[27] reported that glycosides of genistein (isoflavone) and apigenin (flavone) have $[M–H]^-$ ions at *m/z* 431 and these two compounds can only be distinguished by their MS^3 spectrum. MS^n fragmentation of the ion at *m/z* 269 gave a unique product ion (*m/z* 133 for genistein and *m/z* 149 ($^{1,4}B+2H$) for apigenin). According to this information and regarding MS^n data obtained for compound **25**, this was plausibly identified as apigenin-7-*O*-glucoside, since the glycosylation site of flavones is preferential at the 7-OH position. These results were later confirmed by the analysis of a standard solution of apigenin-7-*O*-glucoside under the same LC-DAD/ESI-MS^n conditions.

Compound **31** occurs at a higher retention time ($t_R = 33.7$ min) and was identified as apigenin. It displayed a $[M–H]^-$ ion at *m/z* 269 and, by MS^n fragmentation, the following fragments were observed at *m/z* 225, $[M–H–CO_2]^-$; 201, $[M–H–C_3O_2]^-$; 151, $^{1,3}A$; 149, ($^{1,4}B+2H$). This fragmentation pattern matches the one observed for a standard solution of apigenin.

Identification of a protocatechuic and caffeic acid derivatives (18, 15, 33)

Compound **18** ($t_R = 25.0$ min) showed a molecular ion $[M–H]^-$ at *m/z* 445. The MS^2 spectrum showed the loss of a fragment of 208 Da, due to combined losses of 162 Da and 46 Da. The loss of 46 Da was supported by the formation of a $[M–H–46]^-$ ion at *m/z* 399.

Fragmentation of the ion at *m/z* 237 gave an ion at *m/z* 153 that could possibly be from a protocatechuic acid unit.[28] However, the intensity of this fragment was not enough to perform further fragmentation in order to confirm the presence of protocatechuic acid. Compound **18** was thus speculatively classified as a protocatechuic acid derivative; it is present in trace amounts only in the total plant extract.

Compound **15** ($t_R = 18.2$ min) was identified as a caffeic acid derivative, based on the MS^n pattern of fragmentation. It showed a $[M–H]^-$ ion at *m/z* 625 which when fragmented led to the formation of a product ion at *m/z* 473 (loss of 152 Da). Further fragmentation of this ion produced a MS^3 spectrum with a base peak at *m/z* 341 that corresponds to the loss of 132 Da, probably resulting from neutral loss of a pentose (arabinose, xylose or apiose) or a tartaric acid unit. The ion at *m/z* 341 has already been assigned to caffeic acid hexoside, which was confirmed by the fragment ion at *m/z* 179 [caffeic acid–H]$^-$ obtained in the MS^4 spectrum.[24] It is noteworthy that this compound was not detected in the flowers extract but was present in all the other morphological parts.

Both compounds **32** ($t_R = 26.4$ min) and **33** ($t_R = 27.9$ min) showed $[M–H]^-$ ions at *m/z* 583 and they have similar MS^2 spectra with a base peak at *m/z* 421, resulting from the neutral loss of 162 Da. However, the MS^3 and MS^4 spectra of these two compounds are quite different. For compound **32**, the fragment ion at *m/z* 421 readily loses 162 Da to produce an ion at *m/z* 259, which when fragmented in MS^4 gave a peak at *m/z* 173. The nature of the aglycone could not be determined by these MS^n results only; however, it is clear that there is successive loss of two residues of 162 Da, probably hexosides.

For compound **33**, the MS^3 spectrum of the ion at *m/z* 421 exhibited a base peak at *m/z* 353 and several peaks with high relative intensity at *m/z* 335 (74.5%), 259 (72.5%), 179 (43.2%) and 173 (53.6%). The MS^4 spectrum of the fragment at *m/z* 353 exhibited as base peak a fragment at *m/z* 179 and a very intense peak at *m/z* 173 (95.7% of the base peak). The fragment ion at *m/z* 179 indicates the presence of a caffeic acid derivative but no other identification can be performed based on the available data. Therefore, compound **33** was characterized as a caffeic acid hexoside derivative.

Unidentified compounds (2, 3, 6, 23, 34)

Other peaks were observed and denominated as compounds **2**, **3**, **6**, **23** and **34**. However, the elucidation of their structures based solely on MS^n data has not been completely reached yet.

At a retention time of 7.5 min we observed an intense peak that exhibited a $[M–H]^-$ ion at *m/z* 429. The MS^2 spectrum showed an ion at *m/z* 393, resulting from the loss of 36 Da. MS^n fragmentation gave ions at *m/z* 149 (loss of 244 Da) and 131 (loss of 18 Da due to a molecule of water). This peak was designated as compound **6** and showed three maximum absorptions at 230–245, 280–300 and 340 nm. Nevertheless, it was not possible to identify its structure. It must be mentioned that this compound was found in all plant extracts with the exception of the stems extract.

Compound **23** ($t_R = 34.8$ min) gave a $[M–H]^-$ ion at *m/z* 331 and additional fragmentation formed an ion at *m/z* 155 which corresponds to the loss of 176 Da (probably a glucuronide residue). The MS^3 and MS^4 spectra showed sequential losses of 15 Da that indicates the presence of methyl groups.

CONCLUSIONS

A simple and sensitive LC-DAD/ESI-MS^n method has been used for the comprehensive separation and identification of phenolic compounds in different morphological parts of *Helichrysum devium*. Abundant $[M–H]^-$ ions were observed in ESI-MS^n negative mode, and were used to identify molecular masses of the detected compounds. A total of 34 compounds found in the total aerial parts, leaves, flowers and stems were characterized or tentatively identified based on the MS^n fragmentation behaviour, UV spectra and retention times. Positive identification was facilitated for three of these compounds using authentic standards.

Quinic acid derivatives were found to be the major constituents of *Helichrysum devium* extracts analyzed. A 206 Da neutral loss from $[M–H]^-$ ions of malonylcaffeoylquinic acid isomers was explored for the first time by our LC-DAD/ESI-MS^n method, and indicated that the malonyl group is attached to one caffeoyl group rather than being linked to the quinic acid structure.

The flowers extract revealed the presence of a much higher variety of phenolic compounds, namely flavonoids, most of them as glycosides and/or esterified with acyl groups. A large number of compounds were described for the first time in *Helichrysum* species using LC/MS^n as an analytical tool. The antimicrobial and antioxidant properties of these extracts have been investigated and will be reported elsewhere.

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