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Characterisation of phenolic acid derivatives and flavonoids from different morphological parts of *Helichrysum obconicum* by a RP-HPLC–DAD-(−)–ESI-MS^n method

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ABSTRACT

The phenolic composition from different morphological parts of *Helichrysum obconicum* was investigated for the first time and 50 different phenolic compounds were detected. Phenolic acid conjugates, mainly mono- and di-caffeoylquinic acid derivatives, were the major components; some flavonoid derivatives were also detected in small amounts. Their separation and identification was performed by a high-performance liquid chromatography/electron spray ionisation tandem ion trap mass spectrometry method, with special emphasis on MS^n fragmentation. The presence of di- and tricaffeoylshikimic acid isomers in *Helichrysum* species extracts was reported for the first time, the spectra of these compounds were mainly characterised by the presence of a [caffeoylshikimic acid-H]$^-$ ion at *m/z* 335. A lamiridosins-di-*O*-hexoside, an unusual component in *Asteraceae* species, was also detected.

1. Introduction

The vascular flora of Madeira Archipelago is exuberant and diverse, comprising over 1220 species of which 10% are endemic. Amongst this vast number of plants, a large variety is still used in the local traditional medicine.

Four endemic species of the *genus Helichrysum* (*Asteraceae*) are known and the use of the three abundant ones in traditional medicine is well reported (Rivera & Obón, 1995). One of these endemic species is *Helichrysum obconicum* DC. E; its aerial parts are used as herbal tea for curing digestive, stomachic and intestinal diseases. Recently, chefs from high class hotels have shown interest of introducing this new flavour as a neutraceutical in herbal teas and salads.

The main classes of compounds usually found in *Helichrysum* species are phenolic compounds, coumarins and terpenoids (Al-Rehaily, Albishi, El-Olemy, & Mossa, 2008).

The phenolic composition of two other *Helichrysum* species endemic from Madeira has been investigated before by our group and flavonoids and phenolic acid derivatives were found to be the major bioactive constituents (Gouveia & Castilho, 2009, 2010).

Phenolic compounds are a heterogeneous group of secondary metabolites in vascular plants, vital for normal plant development. They are known to be responsible for the colour and flavour of many plants but their main interest relies on their biological properties such as antioxidant activity, protection against cancer, cardiovascular and neurodegenerative diseases.

The main subclasses of phenolic compounds are phenolic acids and flavonoids. The phenolic acids are mostly hydroxybenzoic acids and hydroxycinnamic acids derived from benzoic and cinnamic acids, respectively (Mattila & Kumpulainen, 2002). Flavonoids normally occur as flavonoid *O*-glycosides or *C*-glycosides and, in some cases, additional hydroxylation, acylation and/or methylation occur(s) (Cuyckens & Claeys, 2004).

The interest in the analysis and identification of the phenolic compounds and their derivatives present in medicinal plant extracts rises not only from the need to find new sources for these compounds but also to establish a relationship between type/structure of the compounds and the extract uses in traditional medicine.

Reversed phase high-performance liquid chromatography (RP-HPLC) hyphenated with mass spectrometry techniques using APCI and ESI interfaces has proved to be a powerful tool for the analysis of plant extract composition. The use of ESI operating in the negative mode (ESI$^-$) has demonstrated to be more selective and efficient in the characterisation of phenolic compounds even those present in trace amounts (Gouveia & Castilho, 2009).

The use of combined techniques allowed for the characterisation of 50 compounds in *H. obconicum*. Most of them are phenolic acid derivatives formed by the esterification of caffeic acid and quinic acid or shikimic acid. They are all described for the first time in this plant.

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In this study, the different morphological parts of the plant used in traditional medicine were analysed in order to establish if there is or not a preferential accumulation of phenolic compounds in one or more parts.

The occurrence of quinic acid derivatives in polar extracts of *Helichrysum* species was reported before (Gouveia & Castilho, 2009), (Carini, Aldini, Furlanetto, Stefani, & Facino, 2001) but not the presence of shikimic acid derivatives.

2. Experimental

2.1. Chemical and standards

HPLC grade acetonitrile (CH_3CN) (Lab-Scan, 99%; Gliwice, Poland), ultra-pure water (Milli-Q Waters, EUA) and formic acid (analytical grade, Merck, Germany) were used for mobile phase preparation in the LC–MS analysis. The methanol used for extraction of *H. obconicum* was AR grade, purchased from Fisher (Lisbon, Portugal). Eluents prepared for LC–MS analysis were additionally filtered through 0.45 μm Nylon membranes (Millipore).

Reference substances apigenin (>99%, HPLC), quercetin (>99%, HPLC) and quercetin-3-*O*-glucoside were purchased from Extrasynthese (Lyon, France) and 5-*O*-caffeoylquinic acid, kaempferol from Acros Organics (Geel, Belgium). 1,3-*O*-dicaffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and 3,4,5-*O*-tricaffeoylquinic acid were obtained from Chengdo biopurity phytochemicals, Lts China (Sichuan, China).

Stock solutions of these compounds (100 μg/mL) were prepared in ethanol and stored in a refrigerator at −20 °C until us for further analys by HPLC–DAD–ESI-MS^n.

2.2. Plant material and sample preparation

Samples of *H. obconicum* were collected by us from the wild in the North Coast of Madeira Island. The collected plant material consisted of total aerial parts and part of it was separated into leaves, flowers and stems for individual analysis. However, the amount of collected flowers was not sufficient to be analysed. The plants were authenticated by taxonomist Fátima Rocha and a voucher was deposited in the Madeira Botanical Garden herbarium collection.

Dried and powdered plant material (100 g) was exhaustively extracted by maceration with methanol (1 L), 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). Stock solutions with concentrations (m/v) of 5 mg/mL were prepared by dissolving dried extract in initial HPLC mobile phase aqueous formic acid 0.1% (v/v) – CH_3CN (20:80, v/v).

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

2.3. HPLC–DAD–ESI-MS^n analysis

2.3.1. Liquid chromatography

The HPLC system consisted of a Dionex ultimate 3000 series instrument (California, EUA) coupled to a binary pump, a diode-array detector (DAD), an autosampler and a column compartment. UV–visible spectra were recorded from 210 to 520 nm and chromatograms were 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). The eluting conditions applied were: 0–10 min, linear gradient from 20% to 25% A; 10–20 min, 25% A isocratic; 20–40 min, linear gradient from 25% to 50% A; 42–47 min, linear gradient from 50% to 100% A for washing, return to 20% A at 55 min, and finally 5 min isocratic to re-equilibrate the column. The mobile phase flow rate was 0.4 mL/min and column temperature was controlled at 30 °C.

2.3.2. Mass spectrometry

The HPLC system described above was coupled on-line to a Bruker Esquire (Bremen, Germany) model 6000 ion trap mass spectrometer fitted with an ESI source. Data acquisition and processing were performed using Esquire control software. Negative 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 rate of 10.0 mL/min and as a nebulising gas at a pressure of 50 psi. The nebuliser temperature was set at 365 °C and a potential of + 4500 V was used on the capillary. Ultrahigh-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 with *auto* MS^n mode, with isolation width of 4.0 *m/z*. For MS^n analysis, mass spectrometer was scanned from 10 to 1000 *m/z* with fragmentation amplitude of 1.0 V (MS^n up to MS^4) and two precursor ions.

3. Results and discussion

The HPLC base peak chromatograms (BPC) in the negative mode profiles of the *H. obconicum* total aerial parts, leaves and stems methanolic extracts are presented in Fig. 1.

The HPLC conditions used allowed for a good separation of a large percentage of compounds and no variation was observed in the three analysis of each sample.

Compounds were characterised based on their HPLC retention time, UV spectra and mostly on their MS^n fragmentation behaviour due to the lack of reference standards for the majority of the extract components.

Most of the detected compounds showed similar UV absorptions maxima with two bands at 230–240 nm and 320–330 nm and a shoulder at 290–300 nm. This type of UV spectra is characteristic of hydroxycinnamic acids. Some peaks with characteristic UV absorptions band for flavonoids were also detected (Mabry, Markham, & Thomas, 1970).

The first approach to the MS^1 spectrum was to identify the deprotonated molecular ion $[M-H]^-$. Generally, for the detected phenolic compounds, the $[M-H]^-$ ion corresponds to the most intense peak in the MS^1 spectra.

Whenever isomers already found on endemic *Helichrysum* species of Madeira Archipelago were detected, their differentiation was achieved based on previous reports where the same analytical method was applied. The next subsections present the description of the characterisation of the observed compounds which were grouped in two types: phenolic acids and flavonoids. The compounds were numbered by their order of elution.

The main fragments observed in the MS^n experiments (n = 1–4) are given in Table 1 and the structures of the compounds found in *H. obconicum* are shown in Fig. 2.

3.1. Phenolic acid derivatives

Amongst the components of *H. obconicum* extracts, there were several phenolic acids derivatives such as the esters of caffeic and quinic or shikimic acids. For the majority of the detected compounds the deprotonated molecular ion, $[M-H]^-$, had

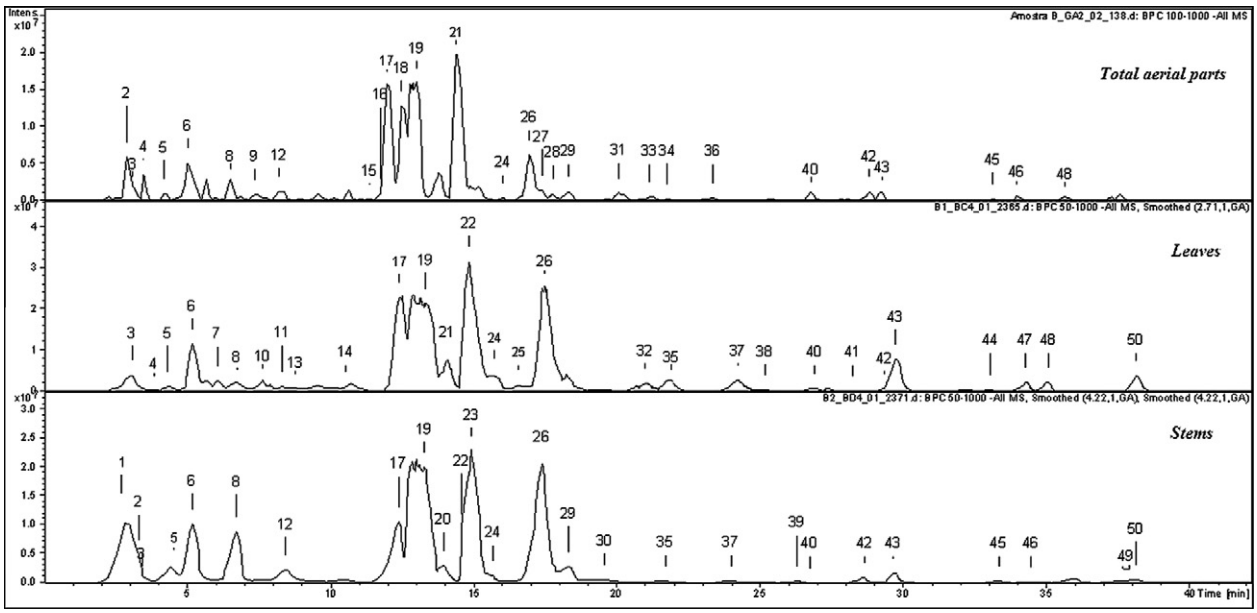


Fig. 1. HPLC–DAD–ESI-MS^n chromatograms (Base Peak Chromatograms – BPC) of *Helichrysum obconicum* methanolic extracts: total aerial parts, leaves and stems.

sufficient intensity to be submitted to subsequent MS^n fragmentation. The loss of the substitution groups are referred to the [M−H]$^-$ ion.

3.1.1. Quinic acid

The presence of quinic acid derivatives on plants from the *Helichrysum* genus has been already reported (Gouveia & Castilho, 2009).

Compound **3** (t_R = 3.1 min) showed a [M−H]$^-$ ion at *m/z* 191 and its MS^2 fragmentation gave a fragment ion at *m/z* 127 characteristic of quinic acid.

3.1.2. Caffeoylquinic acid

MS^n fragmentation experiments of caffeoylquinic acid isomers are well studied and the locations of the caffeoyl groups in the quinic acid structure can be determined taking into account the relative intensities of characteristics MS^n ions (Clifford, Knight, & Kuhnert, 2005).

Two compounds **5** (t_R = 4.3 min) and **6** (t_R = 5.0 min) gave a [M−H]$^-$ ion at *m/z* 353 and were detected in all extracts. The MS^2 spectrum of compound **5** (t_R = 4.3 min) showed a fragment ion at *m/z* 191 as base peak and an intense ion at *m/z* 179 (>40% of base peak). Based on the hierarchical key proposed by Clifford et al. (2005) compound **5** was characterised as 3-*O*-caffeoylquinic acid.

Compound **6** was identified as 5-*O*-caffeoylquinic acid by comparing its UV and MS^n spectra and HPLC retention time with those of a reference standard.

Besides the monocaffeoylquinic acids isomers, there are six dicaffeoylquinic acid derivatives in *H. obconicum*.

Compounds **8** (t_R = 6.6 min), **17** (t_R = 12.1 min), **18** (t_R = 12.5 min), **19** (t_R = 13.2 min), **22** (t_R = 14.7 min) and **31** (t_R = 20.1 min) all exhibited [M−H]$^-$ ions at *m/z* 515. MS^2 fragmentation of the ion at *m/z* 515 gave a fragment ion at *m/z* 353, as base peak, suggesting the loss of a caffeoyl residue (162 Da).

They were identified by comparison with standard compounds as 1,3-*O*-dicaffeoylquinic acid (compound **8**), 3,4-*O*-dicaffeoylquinic acid (compound **17**), 1,5-*O*-dicaffeoylquinic acid (compound **18**), 3,5-*O*-dicaffeoylquinic acid (compound **19**) and 4,5-*O*-dicaffeoylquinic acid (compound **22**).

The occurrence of dicaffeoylquinic acid isomers **8**, **17**, **18** and **19** in *Helichrysum* species has been reported before, namely in our recent work on endemic *Helichrysum* species from Madeira Archipelago where the completely fragmentation characterisation is presented (Gouveia & Castilho, 2009).

Compound **31**, which is a 4-OH substituted quinic acid (MS^3 spectrum base peak at *m/z* 173) showed a MS^2 ion at *m/z* 299 and was identified as 1,4-*O*-dicaffeoylquinic acid (Clifford et al., 2005).

Two compounds **13** (t_R = 8.8 min) and **43** (t_R = 29.7 min) displayed the same deprotonated molecular ion at *m/z* 677.

Compound **43** was found in all morphological parts of the plant and its MS^n fragmentation showed three consecutive losses of caffeoyl moieties (162 Da) which are consistent with those found for a standard solution of 3,4,5-tricaffeoylquinic acid and comparing to literature reports (Gouveia & Castilho, 2009).

Despite the fact that compound **13** gave the same [M−H]$^-$ ion as compound **43**, it occurred at a much shorter retention time. In the MS^2 spectrum, a neutral loss of 162 Da was also observed forming a fragment ion at *m/z* 515. This 162 Da residue can be associated to a hexoside group rather than to a caffeoyl group, justifying the low retention time of this compound. The presence of a MS^3 ion at *m/z* 335 (>30% of base peak) and a strong MS^4 ion at *m/z* 179 (>80% of base peak) indicates a 1,3-*O*-dicaffeoylquinic acid hexoside.

The hexoside group must be linked to one of the caffeoyl groups since an intense fragment ion [caffeic acid + hexoside-H]$^-$ at *m/z* 341 was observed. However, it was not possible to identify which of the caffeoyl group is glycosylated. Thus, compound **13** was assigned as 1,3-*O*-dicaffeoylquinic acid hexoside.

Compound **42** (t_R = 29.1 min) showed a [M−H]$^-$ ion at *m/z* 557 and was found in all analysed extracts. Its MS^2 spectrum gave a fragment ion at *m/z* 395 due to the loss of a 162 Da residue. Fragmentation of the MS^2 ion at *m/z* 395 gave a fragment ion at *m/z* 233, reported before as being acetylquinic acid (Gouveia & Castilho, 2009). These two successive losses of 162 Da and the presence of the ion at *m/z* 233 indicate a dicaffeoylquinic acid structure substituted with an acyl group. The loss of a caffeoyl-acyl residue was not observed, so it is possible to deduce that the acyl group is directly connected to the quinic acid structure. However, with

Table 1
Characterisation of phenolic compounds of the methanolic extract of total aerial parts, leaves and stems from *Helichrysum obconicum* by HPLC–DAD–ESI-MS^n.

No.	t_R (min)	UV λ_{max} (nm)	$[M-H]^-$ m/z	HPLC–DAD–ESI-MS^n m/z (% base peak)	Assigned identity	Morphological part
1	2.7	225, 276, 300	487	MS^2 [487]: 341 (100), 251 (10.0), 179 (52.2), 145 (98.6) MS^3 [487 → 341]: 179 (100), 161 (11.9), 143 (24.0), 113 (16.7), 101 (16.6) MS^4 [487 → 341 → 179]: 118 (32.0), 111 (100), 89 (72.6)	Caffeic acid-*O*-hexoside-*O*-rhamnoside	Stems
2	2.8	–	683	MS^2 [683]: 342 (10.8), 341 (100) MS^3 [683 → 341]: 179 (100), 161 (14.2), 143 (18.3), 119 (20.4), 113 (20.3) MS^4 [683 → 341 → 179]: 119 (60.3), 113 (32.7), 106 (100), 101 (45)	Dimer of caffeic acid-*O*-hexosie	Total aerial parts Stems
3	3.1	–	191	MS^2 [191]: 173 (69.4), 171 (32.7), 155 (35.2), 127 (100), 111 (34.5), 109 (30.8) MS^3 [191 → 127]: 110 (33.6), 109 (100)	Quinic acid	Total aerial parts Leaves Stems
4	3.5	254, 292	317	MS^2 [317]: 225 (100), 207 (13.1), 165 (31.5), 125 (25.8) MS^3 [317 → 225]: 207 (81.5), 125 (91.2), 165 (100)	Unknown	Total aerial parts Leaves
5	4.2	243, 296, 324	353	MS^2 [353]: 191 (100), 179 (37.8), 173 (2.7), 135 (13.9) MS^3 [353 → 191]: 173 (51.7), 127 (100), 111 (40.5), 109 (25.1) MS^4 [353 → 191 → 127]: 109 (100)	3-*O*-Caffeoylquinic acid	Total aerial parts Leaves Stems
6*	5.0	242, 300, 325	353	MS^2 [353]: 191 (100), 179 (3.5), 135 (1.7) MS^3 [353 → 191]: 173 (53.7), 127 (100), 111 (23.6), 109 (20.7) MS^4 [353 → 191 → 127]: 109 (100)	5-*O*-Caffeoylquinic acid	Total aerial parts Leaves Stems
7	6.0	272, 354	609	MS^2 [609]: 447 (31.7), 429 (7.81%), 285 (100), 283 (29.6), 257 (46.8) MS^3 [609 → 285]: 257 (46.8), 255 (100), 219 (15.8), 213 (95.5), 163 (56.0) MS^4 [609 → 285 → 255]: 135 (100)	Kaempferol-di-*O*-hexoside	Leaves
8*	6.5	232, 303, 321	515	MS^2 [515]: 353 (100), 335 (33.8), 191 (24.6), 179 (33.9) MS^3 [515 → 353]: 191 (100), 179 (45.2), 135 (17.4) MS^4 [515 → 353 → 191]: 173 (64.9), 127 (100), 111 (50.0), 109 (26.0)	1,3-*O*-Dicaffeoylquinic acid	Total aerial parts Leaves Stems
9	7.3	–	533	MS^2 [533]: 515 (11.2), 371 (100), 353 (21.3), 335 (4.5), 173 (5.5) MS^3 [533 → 371]: 353 (100), 191 (40.7), 179 (24.6), 173 (33.7) MS^4 [533 → 371 → 353]: 191 (100), 179 (38.5), 173 (18.4), 135 (12.2)	Caffeic acid-*O*-hexoside derivative	Total aerial parts
10	7.7	216, 320	533	MS^2 [533]: 372 (16.4), 371 (100), 353 (17.0) MS^3 [533 → 371]: 353 (100), 191 (45.4), 173 (53.4), 135 (76.9) MS^4 [533 → 371 → 353]: 191 (96.8), 179 (100), 173 (68.6), 135 (12.2)	Caffeic acid-*O*-hexoside derivative	Leaves
11	8.1	306	337	MS^2 [337]: 191 (100) MS^3 [337 → 191]: 173 (33.2), 127 (100), 125 (72.6), 110 (87.0), 93 (55.4)	5-*O*-*p*-Coumaroylquinic acid	Leaves
12	8.4	232, 280	567	MS^2 [567]: 342 (22.2), 341 (100), 330 (31.9), 329 (88.2) MS^3 [567 → 341]: 327 (12.9), 326 (100), 311 (13.8) MS^4 [567 → 341 → 327]: 311 (100)	Dimethoxylflavanone derivative	Total aerial parts Stems
13	8.8	255, 318	677	MS^2 [677]: 516 (21.5), 515 (100), 353 (15.6) MS^3 [677 → 515]: 353 (100), 341 (54.0), 335 (37.4), 323 (17.8), 179 (80.2) MS^4 [677 → 515 → 353]: 191 (100), 179 (86.5)	1,3-*O*-Dicaffeoylquinic acid hexoside	Leaves
14*	10.1	258, 353	463	MS^2 [463]: 301 (100), 300 (19.4) MS^3 [463 → 301]: 271 (49.2), 255 (20.8), 179 (100), 175 (17.0), 151 (62.7) MS^4 [463 → 301 → 179]: 169 (100), 151 (94.8)	Quercetin-3-*O*-glucoside.	Leaves
15	11.4	–	415	MS^2 [415]: 371 (5.6), 179 (100), 161 (29.0), 143 (12.1) MS^3 [415 → 179]: 143 (59.5), 119 (100), 113 (58.0)	Caffeic acide derivative	Total aerial parts
16	11.7	262, 351	451	MS^2 [451]: 244 (16.2), 243 (100), 199 (1.8) MS^3 [451 → 243]: 211 (53.8), 199 (100), 143 (89.5), 123 (36.1) MS^4 [451 → 243 → 199]: 184 (100)	Unknown	Total aerial parts
17*	12.1	243, 300, 324	515	MS^2 [515]: 353 (100), 335 (8.6), 179 (32.2), 173 (39.5) MS^3 [515 → 353]: 191 (41.7), 179 (48.0), 173 (100), 135 (8.4) MS^4 [515 → 353 → 173]: 155 (75.3), 111 (100), 109 (31.0)	3,4-*O*-Dicaffeoylquinic acid	Total aerial parts Leaves Stems

18*	12.5	242, 300, 327	515	MS^2 [515]: 353 (100), 335 (8.8), 191 (54.2) MS^3 [515 → 353]: 191 (100) MS^4 [515 → 353 → 191]: 173 (23.0), 127 (100), 111 (79.7), 109 (36.1)	1,5-*O*-Dicaffeoylquinic acid	Total aerial parts
19*	13	242, 298, 326	515	MS^2 [515]: 353 (100), 191 (11.9) MS^3 [515 → 353]: 191 (100), 179 (39.6), 135 (7.8) MS^4 [515 → 353 → 191]: 173 (90.1), 127 (100), 111 (63.4)	3,5-*O*-Dicaffeoylquinic acid	Total aerial parts Leaves Stems
20	13.9	244, 297, 326	601	MS^2 [601]: 557 (30.7), 516 (39.8), 515 (95.5), 439 (15.3), 395 (100), 377 (21.8) MS^3 [601 → 395]: 335 (48.4), 233 (100), 179 (51.8), 173 (58.1) MS^4 [601 → 395 → 233]: 173 (100)	Malonyl-1,4-*O*-Dicaffeoylquinic acid	Stems
21	14.5	243, 299, 326	601	MS^2 [515]: 353 (100), 179 (10.0), 173 (28.2) MS^3 [515 → 353]:191 (23.7), 179 (67.7), 173 (100), 135 (11.3) MS^4 [515 → 353 → 173]: 155 (43.8), 111 (100)	Malonyl-3,4-*O*-dicaffeoylquinic acid	Total aerial parts Leaves
22*	14.7	245, 297, 327	515	MS^2 [515]: 353 (100), 299 (11.5), 203 (20.4), 179 (19.9), 173 (22.8) MS^3 [515 → 353]: 191 (20.6), 179 (53.9), 173 (100), 135 (10.3) MS^4 [515 → 353 → 191]: 127 (14.0), 109 (12.3), 93 (100)	4,5-*O*-Dicaffeoylquinic acid	Leaves Stems
23	15.0	–	601	MS^2 [601]: 557 (62.5), 515 (100), 395 (82.3), 233 (32.4) MS^3 [601 → 515]: 354 (13.4), 353 (100), 335 (14.1), 191 (82.1) MS^4 [601 → 515 → 353]: 192 (16.9), 191 (100)	Malonyl-1,5-*O*-dicaffeoylquinic acid	Stems
24	15.7	266, 332	445	MS^2 [455]: 281 (31.7), 270 (20.9), 269 (100), 175 (12.3) MS^3 [455 → 269]: 225 (92.3), 224 (23.3), 201 (26.6), 151 (42.5), 149 (100) MS^4 [445 → 269 → 149]: 107 (100)	Apigenin-7-*O*-glucuronide	Total aerial parts Leaves Stems
25	16.6	–	499	MS^2 [499]: 353 (100), 337 (27.1), 335 (37.2), 319 (28.6), 179 (22.1), 173 (61.8) MS^3 [499 → 353]: 191 (73.9), 179 (51.0), 173 (100), 135 (30.1) MS^4 [499 → 353 → 173]: 111 (100), 93 (58.7)	3-*O*-*p*-coumaroyl-4-*O*-caffeoylquinic acid	Leaves
26	17	243, 300, 326	601	MS^2 [601]: 557 (50.9), 395 (100) MS^3 [601 → 395]: 233 (100), 173 (78.3) MS^4 [601 → 395 → 233]: 173 (100)	Malonyl-4,5-*O*-dicaffeoylquinic acid	Total aerial parts Leaves Stems
27	17.5	323	499	MS^2 [499]: 338 (13.6), 337 (100), 335 (6.8), 173 (14.4), 163 (24.0) MS^3 [499 → 337]: 191 (6.3), 173 (44.8), 163 (100), 119 (10.1) MS^4 [499 → 337 → 163]: 120 (5.7), 119 (100), 118 (4.8)	3-*O*-Coumaroyl-5-*O*-caffeoylquinic acid	Total aerial parts
28	17.8	–	499	MS^2 [499]: 354 (17.2), 353 (100), 337 (9.9), 191 (4.6), 179 (3.7) MS^3 [499 → 353]: 191 (100), 179 (47.1), 173 (3.5), 135 (14.5) MS^4 [499 → 353 → 191]: 173 (100), 171 (51.6), 127 (99.2), 111 (35.5), 109 (39.6)	3-*O*-Caffeoyl-5-*O*-coumaroylquinic acid	Total aerial parts
29	18.3	–	625	MS^2 [625]: 474 (16.1), 473 (100), 293 (8.6) MS^3 [625 → 473]: 342 (8.7), 341 (100), 293 (51.1), 233 (22.9), 179 (20.7) MS^4 [625 → 473 → 341]: 239 (46.4), 179 (100), 164 (21.9)	Caffeic acid derivative	Total aerial parts Stems
30	19.6	–	687	MS^2 [687]:643 (15.9), 601 (100), 597 (40.4), 557 (61.4), 437 (39.6) MS^3 [687 → 601]: 557 (65.6), 515 (15.0), 395 (100), 353 (31.1) MS^4 [687 → 601 → 395]: 233 (100)	*O*-Dimalonyl-*O*-dicaffeoylquinic acid	Stems
31	20.1	–	515	MS^2 [515]: 354 (17.7), 353 (100), 191 (8.2), 179 (7.3), 173 (12.1) MS^3 [515 → 353]: 191 (68.8), 179 (73.3), 173 (100), 135 (9.3) MS^4 [515 → 353 → 173]: 155 (100), 111 (75.6)	1,4-*O*-Dicaffeoylquinic acid	Total aerial parts
32	21.0	247, 300, 326	499	MS^2 [499]: 338 (12.5), 337 (100), 335 (2.96), 173 (27.0) MS^3 [499 → 337]: 173 (100), 163 (43.1) MS^4 [499 → 337 → 173]: 111 (100), 93 (31.3)	4-*O*-*p*-coumaroyl-5-*O*-caffeoylquinic acid	Leaves
33	21.1	–	497	MS^2 [497]: 337 (75.0), 335 (82.1), 255 (20.9), 179 (100), 173 (32.5) MS^3 [497 → 179]: 136 (2.6), 135 (100)	Dicaffeoylshikimic acid	Total aerial parts
34	21.7	326	529	MS^2 [529]: 368 (16.2), 367 (100), 179 (11.2), 161 (9.5) MS^3 [529 → 367]: 179 (100); 173 (23.4), 161 (54.9), 135 (79.8) MS^4 [529 → 367 → 179]: 135 (100)	Caffeic acid derivative	Total aerial parts
35	21.8	–	497	MS^2 [497]: 353 (45.7), 337 (29.6), 335 (63.5), 211 (27.3), 179 (100)	Dicaffeoylshikimic acid	Leaves

(*continued on next page*)

Table 1 (*continued*)

No.	t_R (min)	UV λ_{max} (nm)	$[M-H]^-$ *m/z*	HPLC–DAD–ESI-MS^n *m/z* (% base peak)	Assigned identity	Morphological part
				MS^3 [497 → 335]: 179 (100), 161 (48.6), 135 (24.1) MS^3 [499 → 173]: 136 (14.2), 135 (100)		Stems
36	23.4	–	497	MS^2 [497]: 335 (22.5), 317 (48.8), 273 (100), 255 (36.0), 211 (46.2), 179 (35.1), 161 (32.9) MS^3 [497 → 273]: 255 (49.8), 229 (100), 211 (82.0), 179 (48.0), 159 (50.6) MS^4 [497 → 273 → 229]: 168 (100)	Unknown	Total aerial parts
37	23.9	265	497	MS^2 [497]: 351 (28.8), 317 (100), 291 (34.8), 273 (83.1), 255 (43.0), 231 (32.1), 211 (44.8), 179 (50.6) MS^3 [497 → 317]: 274 (65.6), 256 (37.1), 186 (100) MS^4 [497 → 273 → 229]: 168 (100)	Unknown	Leaves Stems
38	25.1	–	585	MS^2 [585]: 543 (28.5), 541 (100), 395 (37.7), 379 (14.5) MS^3 [585 → 541]: 396 (17.9), 395 (78.7), 379 (100) MS^4 [585 → 541 → 379]: 233 (100), 229 (18.1), 173 (26.7), 163 (56.9)	Caffeoyl-*O*-(malonyl)-*O*-coumaroylquinic acid	Leaves
39	26.1	216, 287	341	MS^2 [341]: 327 (17.1), 326 (100) MS^3 [341 → 326]: 311 (100) MS^4 [341 → 326 → 311]: 283 (100), 281 (25.6), 267 (46.4)	Dimethoxylflavanone	Stems
40	26.8	–	425	MS^2 [425]: 179 (100), 135 (35.9) MS^3 [425 → 179]: 135 (100)	Caffeic acid derivative	Total aerial parts Leaves Stems
41	28.2	–	445	MS^2 [445]: 179 (100), 135 (64.0) MS^3 [445 → 179]: 135 (100)	Caffeic acid derivative	Leaves
42	29.1	244, 327	557	MS^2 [557]: 396 (14.1), 395 (100), 233 (5.9) MS^3 [557 → 395]: 234 (12.6), 233 (100), 173 (81.5) MS^4 [557 → 395 → 233]: 173 (100)	Acetyldicaffeoylquinic acid	Total aerial parts Leaves Stems
43*	29.2	245, 291, 327	677	MS^2 [677]: 516 (23.2), 515 (100), 353 (13.8) MS^3 [677 → 515]: 353 (100), 335 (8.9), 191 (22.6), 179 (23.8), 173 (59.7) MS^4 [677 → 515 → 353]: 191 (39.6), 179 (74.6), 173 (100), 135 (16.5)	3,4,5-Tri-*O*-caffeoylquinic acid	Total aerial parts Leaves Stems
44	33.0	249	707	MS^2 [707]: 675 (100), 545 (92.0), 353 (45.1), 513 (32.6) MS^3 [707 → 675]: 514 (26.1), 513 (100) MS^4 [707 → 675 → 513]: 495 (25.1), 339 (100)	Unknown	Leaves
45	33.2	–	327	MS^2 [327]: 325 (19.1), 291 (40.5), 229 (100), 211 (64.5), 209 (20.9), 171 (56.7) MS^3 [327 → 229]: 227 (46.7), 211 (100), 165 (95.4), 125 (66.8) MS^4 [327 → 229 → 211]: 135 (100)	Unknown	Total aerial parts Stems
46	34.0	–	583	MS^2 [583]: 421 (100), 259 (6.8) MS^3 [583 → 421]: 259 (100), 173 (76.9) MS^4 [583 → 421 → 259]: 173 (100)	Lamiridosins-di-*O*-hexoside	Total aerial parts Stems
47*	34.2	250, 332	269	MS^2 [269]: 227 (54.4), 226 (38.8), 225 (100), 201 (71.1), 151 (43.2), 149 (61.0) MS^3 [269 → 225]: 181 (100)	Apigenin	Leaves
48	34.7	–	659	MS^2 [659]: 498 (21.1), 497 (100), 479 (20.2), 453 (14.4), 335 (16.8) MS^3 [659 → 497]: 453 (49.3), 335 (100), 317 (65.4), 179 (51.1) MS^4 [659 → 497 → 335]: 179 (100), 135 (67.9), 109 (64.8)	Tri-*O*-caffeoylshikimic acid	Total aerial parts Leaves
49	37.7	–	287	MS^2 [287]: 269 (100), 241 (57.4), 239 (41.4), 171 (39.4), 155 (61.9), 127 (37.2)	Unkown	Stems
50	38.1	–	599	MS^2 [599]: 438 (18.5), 437 (100) MS^3 [599 → 437]: 275 (100), 173 (69.5) MS^4 [599 → 437 → 275]: 173 (100)	Unknown	Leaves Stems

* Compared with a reference standard.

– UV spectra have not been properly observed due to low intensity.

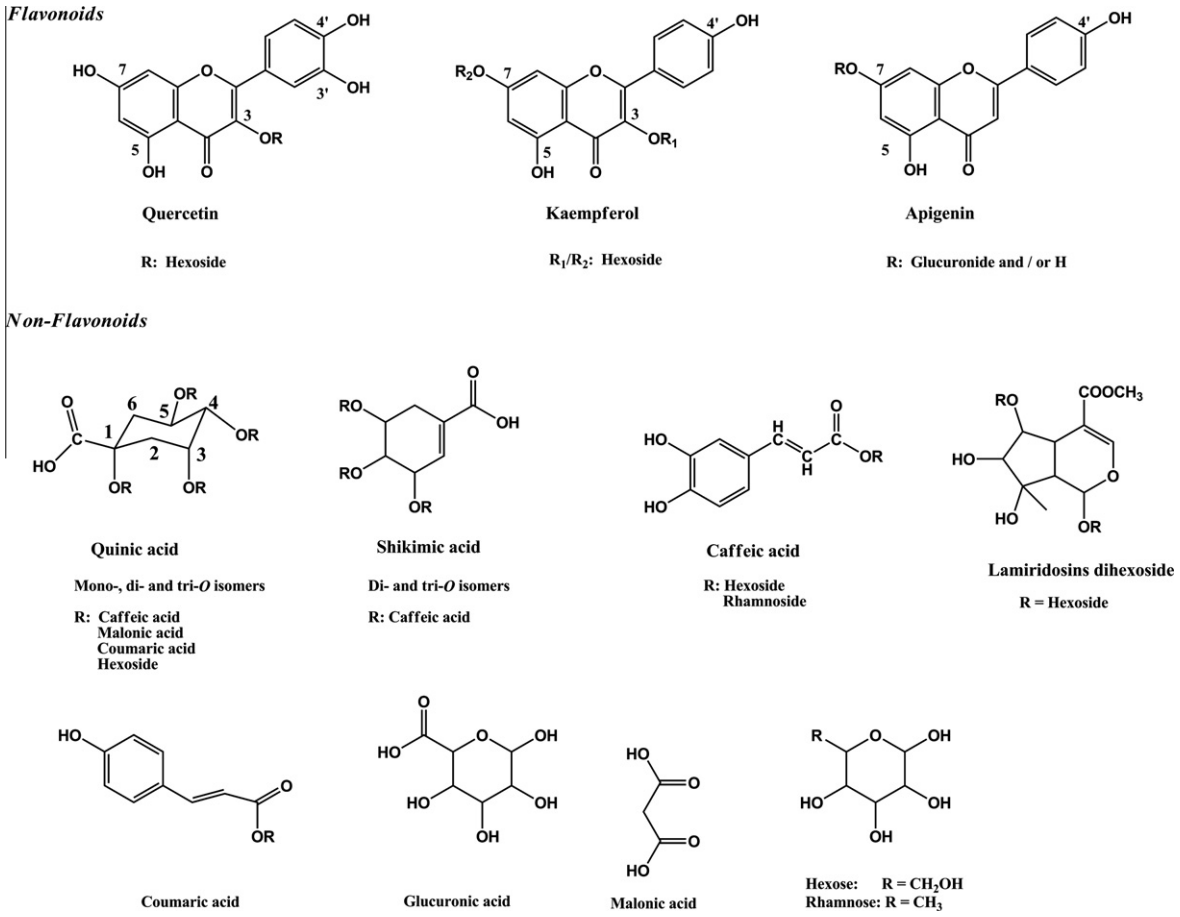


Fig. 2. Chemical structures of the main classes of compounds found in the methanolic extracts of *Helichrysum obconicum*.

no more MS^n data available, compound **42** was tentatively characterised as acetyldicaffeoylquinic acid.

3.1.3. Malonylcaffeoylquinic acid

Five caffeoylquinic acid derivatives containing a malonyl group in their structures were found.

Compounds **20** (t_R = 13.9 min), **21** (t_R = 14.5 min), **23** (t_R = 15.0 min), **26** (t_R = 17.0 min) and **30** (t_R = 19.6 min) displayed a $[M-H]^-$ ion at *m/z* 601 and gave characteristic fragment ions of malonyl $[M-H-44]^-$ at *m/z* 557 and $[M-H-86]^-$ at *m/z* 515 (Zhang, Shi, Qu, & Cheng, 2007). However, their MS^n fragment ions intensities were different and it was possible to characterise them by comparison with literature data.

Compounds **20**, **21** and **26** have been detected in our previous work with another *Helichrysum* species (Gouveia & Castilho, 2009) under the same experimental conditions and were identified as malonyl-1,4-*O*-dicaffeoylquinic acid (compound **20**), malonyl-3,4-*O*-dicaffeoylquinic acid (compound **21**) and malonyl-4,5-dicaffeoylquinic acid (compound **26**).

MS^n fragmentation pattern of compound **23** was different from the isomers described above. MS^2 fragmentation of the ion at *m/z* 601 gave a fragment ion at *m/z* 515, as base peak, which represents the loss of 86 Da. The non observation of a 206 Da loss in the MS^2 spectrum reveals that the malonyl group is directly linked to the quinic acid structure. The dicaffeoylquinic acid residue (*m/z* 515) was submitted to further fragmentation and was characterised based on Clifford's hierarchical key (Clifford et al., 2005). The MS^2 ions at *m/z* 335 and 179 were weak (ca. 1% and 0.71% of base peak, respectively) which matches the 1,5-*O*-dicaffeoylquinic acid structure, as observed for compound **18**. Therefore, compound **23** was characterised as (malonyl)-1,5-*O*-diCQA.

Compound **30** displayed a $[M-H]^-$ ion at *m/z* 687. The MS^2 spectrum gave several fragment ions at *m/z* 643, 557, 437 and, as base peak, a fragment ion at *m/z* 601. The occurrence of fragments at *m/z* 643 and 601 can be associated to the loss of 44 and 86 Da, respectively, which is characteristic of malonyl groups.

MS^3 fragmentation of the ion at *m/z* 601 exhibited a fragment ion at *m/z* 395 (loss of 206 Da) as base peak. This type of fragmentation was described above for dicaffeoylquinic acids with malonyl moieties linked to one of the caffeoyl groups.

In compound **30**, one of the malonyl groups is directly attached to the quinic acid structure and the other is linked to a caffeoyl group. Still, with no further MS^n information, the exact location of the substituent groups in the quinic acid structure is difficult to establish. So, compound **30** was characterised as *O*-dimalonyl-*O*-dicaffeoylquinic acid.

3.1.4. Coumaroylquinic acid

Amongst the above identified quinic acid derivatives, some isomers with a coumaroyl group on their structures could be found.

Compound **11**(t_R = 8.3 min) showed a $[M-H]^-$ ion at *m/z* 337 and was identified as 5-*O*-*p*-coumaroylquinic acid. This identification was achieved by comparison of the main fragment ions formed in the MS^n fragmentation experiments with those described in literature data (Clifford, Johnston, Knight, & Kuhnert, 2003). This compound was only detected in the leaves extract.

Four compounds (**25**, **27**, **28** and **32**) with a deprotonated molecular ion, $[M-H]^-$, at *m/z* 499 were found. They all showed

close retention times which suggests a structural similarity. They were characterised based on the differences found on their MS^n spectra.

For compounds **25** (t_R = 16.6 min) and **28** (t_R = 17.8 min) the MS^2 fragmentation of the MS^1 ion at *m/z* 499 showed the loss of a 146 Da residue (coumaroyl group) giving a caffeoylquinic acid fragment ion at *m/z* 353, as base peak.

For compound **25** the fragmentation of the ion at *m/z* 353 gave fragment ions at *m/z* 173 (base peak), 179 (ca. 50.0% of base peak), 135 (ca. 30.0% of base peak) and 191 (ca. 8% of base peak). The occurrence of a base peak ion at *m/z* 173 indicates the presence of a quinic acid substituted at position 4-OH (Clifford et al., 2005). The exact location of the coumaroyl group is difficult to establish, but comparing our previously identification of coumaroycaffeoylquinic acid isomers and taking into account the results presented by Clifford, Marks, Knight, and Kuhnert (2006) compound **25** was identified as 3-*O*-*p*-coumaroyl-4-*O*-caffeoylquinic acid.

The $[quinic\ acid\text{-}H]^-$ ion at *m/z* 191 appeared in the compound **28** MS^3 spectrum as the base peak. Based on the main fragments detected on MS^n fragmentation and in literature data (Clifford et al., 2006), compound **28** was identified as 3-*O*-caffeoyl-5-*O*-*p*-coumaroylquinic acid.

The other two isomers, compounds **27** (t_R = 17.5 min) and **32** (t_R = 21.0 min), lost first the caffeoyl residue (162 Da) in the MS^2 fragmentation, originating a fragment ion at *m/z* 337. MS^n analysis of this ion revealed a coumaroylquinic acid structure. Compound **27** gave a MS^3 fragment ion at *m/z* 163 and compound **32** a MS^3 fragment ion at *m/z* 173 as MS^3 spectra base peaks.

Based on Clifford et al. (2006) MS^n studies with this class of compounds it was possible to characterise compound **27** as 3-*O*-*p*-coumaroyl,5-*O*-caffeoylquinic acid and compound **32** as 4-*O*-*p*-coumaroyl,5-*O*-caffeoylquinic acid.

Compound **38** (t_R = 25.1 min) displayed a $[M-H]^-$ ion at *m/z* 585 and was only detected in the leaves extract. MS^2 fragmentation of this ion gave a fragment ion at *m/z* 541 due to the loss of 44 Da, which corresponds to a dicarboxylation from a dicarboxylic acid. Additionally, another low intensity MS^2 ion at *m/z* 499 was detected, which corresponds to the loss of 86 Da residue indicating a malonyl moiety. More precisely, this ion at *m/z* 499 can be assigned to a caffeoylcoumaroylquinic acid.

The MS^3 spectrum showed two main fragment ions at *m/z* 379 (base peak) and *m/z* 395 (ca. 94 % of base peak) and were assigned to the loss of caffeoyl (162 Da) and coumaroyl (146 Da) moieties, respectively.

The subsequent fragmentation of these two ions led to a fragment ion at *m/z* 233 that was reported before as being acetylquinic acid (Gouveia & Castilho, 2009).

The loss the caffeoyl group (162 Da) to form the base peak in the MS^3 spectrum indicates that the malonyl group is linked to the coumaroyl group.

The exact location of the substituent groups in the quinic acid structure is difficult to establish based on the available MS^n data. Thus, compound **38** was assigned as caffeoyl-*O*-(malonyl)-*O*-coumaroylquinic acid.

3.1.5. Caffeoylshikimic acids

Two compounds at a retention times of 21.1 min (compound **33**) and 21.8 min (compound **35**) showed a $[M-H]^-$ ion at *m/z* 497. Further MS^n fragmentation of the deprotonated molecular ion was identical for both compounds.

Under MS^2 fragmentation, this ion easily lost 318 Da to form a fragment ion at *m/z* 179, as base peak in the MS^2 spectrum, which suggests the presence of a caffeic acid. The caffeic acid moiety was confirmed with the MS^4 ions at *m/z* 161 and 135 formed from the dissociation the $[caffeic\ acid\text{-}H]^-$ ion, caused by the losses of H_2O and CO_2, respectively (Scheme 1).

Also in the MS^2 spectrum, a strong fragment ion at *m/z* 335 was observed indicating the loss of 162 Da from the deprotonated molecular ion indicating the presence of a caffeic acid residue. MS^n fragmentation of the ion at *m/z* 335 showed the $[caffeic\ acid\text{-}H]^-$ ion at *m/z* 179 and its dissociation fragment ions.

The fragmentation pattern observed for the ion at *m/z* 335 has been previously described in literature for a caffeoylshikimic acid (Fang, Yu, & Prior, 2002; Hokkanen, Mattila, Jaakola, Pirttilaî, & Tolonen, 2009).

Therefore, based on these MS^n data, compounds **33** and **35** were characterised as having two caffeoyl groups and were characterised as dicaffeoylshikimic acids.

Compound **33** was detected only on the total aerial parts extract while compound **35** could be detected in the leaves and stems extracts of *H. obconicum*.

Compound **48** (t_R = 34.9 min) showed a $[M-H]^-$ ion at *m/z* 659 which in MS^2 fragmentation formed a fragment ion at *m/z* 497, representing the loss of a 162 Da residue. MS^n fragmentation of the ion at *m/z* 497 was identical to that found for compounds **33** and **35**, with main fragment ions at *m/z* 335, 179, 161 and 135. The caffeoyl groups must be linked to each of the hydroxyl groups of shikimic acid, since fragment ions related to combined hexoside groups were not detected. Thus, compound **48** was characterised as a tricaffeoylshikimic acid.

Scheme 1 shows the proposed structures for the main fragment ions observed for this tricaffeoylshikimic acid derivative and the MS^n spectra for compound **48** are represented in Fig. 3. Due to the low absorption coefficient of shikimic acid, their UV spectra have not been properly observed.

To our knowledge, this was the first time that shikimic acid derivatives were identified in *Helichrysum* species and they could be found in all extracts of *H. obconicum.*

3.1.6. Caffeic acid derivatives

Compound **1** (t_R = 2.7 min) gave a $[M-H]^-$ ion at *m/z* 487 and was found in the stems of *H. obconicum*. Its MS^2 fragmentation showed the loss of 146 Da resulting in a fragment ion at *m/z* 341. The MS^3 spectrum of this ion showed the loss of a 162 Da residue, resulting in a fragment ion at *m/z* 179 which indicates the presence of a caffeic acid derivative. The two groups linked to the caffeic acid were identified as sugar unites, rhamnoside (146 Da) and a hexoside (162 Da), rather than hydroxycinnamic acids moieties due to the low retention time of compound **1**. Therefore, compound **1** was characterised as caffeic acid-*O*-rhamnoside-*O*-hexoside.

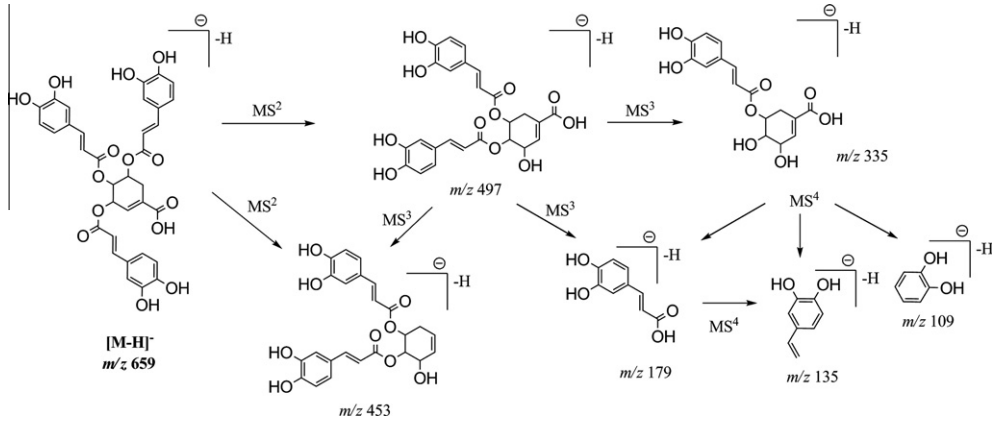
Compound **2** (t_R = 2.9 min) originated a $[M-H]^-$ ion at *m/z* 683 as base peak and also a strong MS^1 ion at *m/z* 341. MS^2 fragmentation of the ion at *m/z* 683 gave a fragment ion at *m/z* 341 confirming that the ion at *m/z* 683 represents a dimer of the ion at *m/z* 341. Fragmentation of this ion (*m/z* 341) gave a fragment ion at *m/z* 179, due to the loss of 162 Da (hexoside residue), which point to a caffeic acid derivative. Thus, compound **2** was assigned as a dimer of caffeic acid *O*-hexoside and was only found in the total aerial parts extract.

Compounds **9** (t_R = 7.3 min) and **10** (t_R = 7.7 min) displayed the same $[M-H]^-$ ion at *m/z* 533. They presented a similar MS^n fragmentation pattern but compound **9** was detected in the total aerial parts and compound **10** in the leaves extract.

Their MS^2 spectra showed a fragment ion at *m/z* 371, as base peak, due to the loss of 162 Da corresponding to a hexoside moiety.

The sequential MS^n fragmentation and the detection of fragment ions at *m/z* 353 and 179 led to the identification of a caffeic acid residue. With no further information available compounds **9** and **10** were assigned as caffeic acid-*O*-hexoside derivatives.

Five other compounds **15**, **29**, **34**, **40** and **41** were detected and also characterised as caffeic acid derivatives. They all exhibited different $[M-H]^-$ ions and distinct MS^n fragmentation behaviour but



Scheme 1. Proposed fragmentation pathway for compound **48** – Tri-*O*-caffeoylshikimic acid.

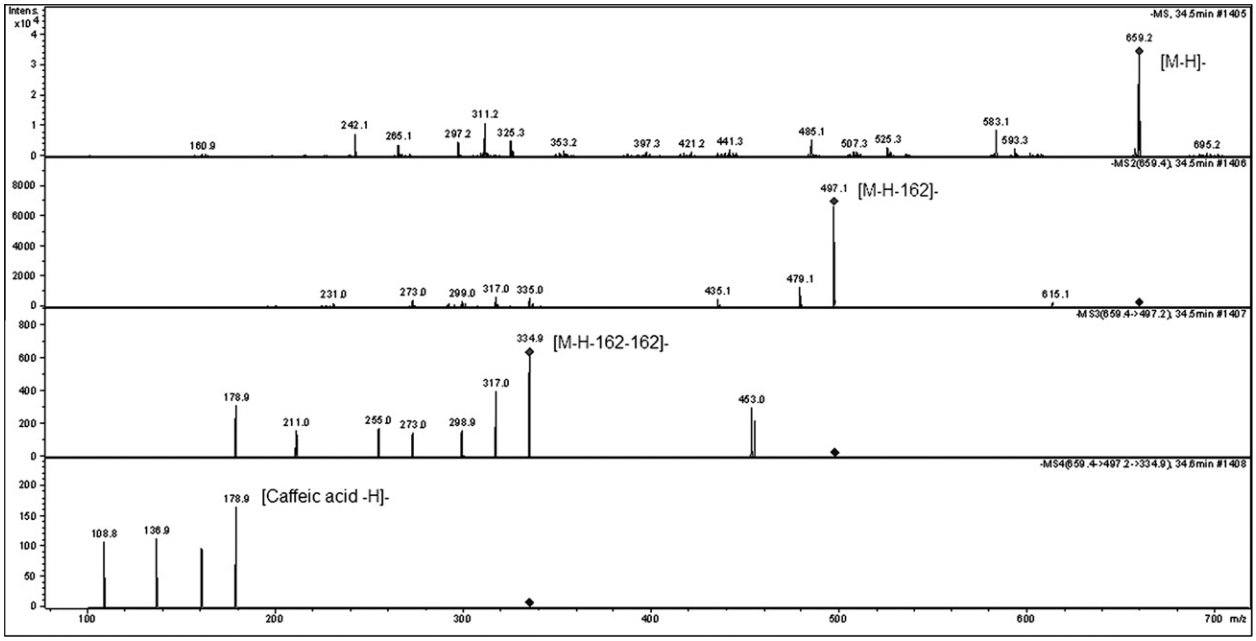


Fig. 3. ESI-MS^n negative mode of compound **48**. Sequential fragmentation, MS^n (n = 2–4), of the ion at m/z 659.

they all had in common a fragment ion at m/z 179 that corresponds to a deprotonated caffeic acid ion, [caffeic acid-H]$^-$.

Compound **15** (t_R = 11.4 min) exhibited a [M−H]$^-$ ion at m/z 415 and was found in the total aerial parts extract. The MS^2 spectrum showed a fragment ion at m/z 179 due to the loss of a 236 Da residue but its nature could not be identified.

Compound **29** (t_R = 18.3 min) showed a [M−H]$^-$ ion at m/z 625 which easily lost a 152 Da residue to form a fragment ion at m/z 473. MS^3 fragmentation of this ion gave a fragment ion at m/z 341 due to the loss of 132 Da residue (could be a pentose or a tartaric acid moiety). The [caffeic acid-H]$^-$ ion was found to be the MS^4 spectrum base peak. There were not observed more fragment ions useful in the identification of the substituent groups. This compound was detected in the total aerial parts and in the stems extract.

Compound **34** (t_R = 21.7 min) exhibited a [M−H]$^-$ ion at m/z 529. Its MS^2 spectrum showed a fragment ion at m/z 367 (loss of 162 Da). The m/z 367 ion indicates the presence of a feruloylquinic acid structure, but it was not possible to detect more fragments to support this idea. The tentatively characterisation of this compound as a caffeic acid derivative results from the observation of a MS^3 ion at m/z 179.

Compound **40** (t_R = 26.8 min) was detected in all extracts and gave a [M−H]$^-$ ion at m/z 425. MS^2 fragmentation showed a loss of 246 Da to form a fragment ion at m/z 179.

Compound **41** (t_R = 28.2 min) displayed a [M−H]$^-$ ion at m/z 445. Its MS^2 spectrum showed a fragment ion at m/z 179 due to the loss of a 266 Da residue. Without any other information, the nature of the neutral losses of 246 Da (compound **40**) and 266 (compound **41**) was not possible to identify and fully characterise these two compounds.

3.2. Flavonoids and lamarosinin-di-O-hexoside

In addition to the phenolic acid derivatives described above, there were also identified a few flavonoid compounds in *H. obconicum*. Almost all flavonoids were found in their glycoside form, containing one or more sugar units, and some were esterified with acyl groups. Free aglycones were found in trace amounts in some samples.

The flavonoid fragment ions were labelled according to the nomenclature proposed by Ma, Li, Heuvel, and Claeys (1997). 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 de C-Ring bonds that have been broken. For conjugated aglycones, Y_0^- is used to refer to the aglycone fragment [M−H-glycoside]$^-$. Compound **7** (t_R = 6.0 min) gave a [M−H]$^-$ ion at *m/z* 609 and its MS^2 fragmentation showed the aglycone ion, Y_0^-, at *m/z* 285 as base peak and also a fragment ion at *m/z* 447 (ca. 31.7% of base peak).

The aglycone ion was formed by the loss of a residue of 324 Da composed of two hexoside moieties (2 × 162 Da). Since the aglycone radical ion was not detected in the MS^2 spectrum and based on the rules reported by Ablajan et al. (2006) this compound was primarily characterised as a flavonoid-*O*-diglycoside.

The MS^3 fragmentation of the ion at *m/z* 285 gave fragment ions at *m/z* 213 (95.5% of base peak), 257 (46.8% of base peak), 163 (56.0% of base peak) and a fragment ion at *m/z* 255 as base peak. These retro-diels–Alder (RDA) fragments are consistent with those found for a standard solution of kaempferol (MS^n fragmentation data not shown).

For flavonols like kaempferol the 7-OH and 3-OH are the most common glycosylation positions; those compounds substituted at position 3-OH should present relative high intensity aglycone radical fragment sometimes higher than the Y_0^- ion (Cuyckens & Claeys, 2005). Such pattern was not observed and the exact location of the hexosides moieties could not be established.

In the MS^2 spectrum there were ions corresponding to the loss of a hexoside moiety (162 Da) at *m/z* 447 and a hexoside plus water moiety (180 Da) at *m/z* 429 and these fragments are associated to the break of a (1 → 2) interglycosidic linkage (Ferreres, Llorach, & Gil-Izquierdo, 2004).

Thus, compound **7** was identified as kaempferol-*O*-(1 → 2)dihexoside and could only be detected in the leaves extract.

Compound **39** (t_R = 26.1 min) displayed a [M−H]$^-$ ion at *m/z* 341. Its UV profile (absorption maximum bands at 216 and 287 nm) suggests a flavanone or a dihydrochalcone skeleton as proposed by Portet et al. (2008). Its MS^2 and MS^3 spectra showed, as base peak, fragment ions at *m/z* 326 and 311 (losses of 15 Da) due to the consecutive radical loss of two methyl groups indicating a methoxylated compound. The MS^4 fragmentation of the ion at *m/z* 311 gave a fragment ion at *m/z* 283 indicating the neutral loss of a CO molecule (28 Da).

The lack of an important loss of H_2O points out a flavanone skeleton (Portet et al., 2008). The ion at *m/z* 283 did not present sufficient intensity to allow further fragmentation in order to fully characterise compound **39**, in particular the position of the substitution groups in the flavanone skeleton. Thus, this compound was tentatively characterised as a dimethoxylflavanone.

Compound **12** (t_R = 8.4 min) showed a [M−H]$^-$ ion at *m/z* 567 and its MS^2 fragmentation formed a fragment ion at *m/z* 341 due to the loss of a 226 Da residue. Further fragmentation of the ion at *m/z* 341 gave similar fragmentation behaviour to that found for compound **39**. The nature of the 226 Da residues could not be determined but it is clear that is must be a hydrophilic group given the low retention time of elution of this compound. Therefore, compound **12** was tentatively characterised as being a dimethoxylflavanone derivative.

Compound **12** was found in the total aerial parts and stems extracts while compound **39** was only detected in the stems extract.

Compound **14** (t_R = 10.1 min) displayed a [M−H]$^-$ ion at *m/z* 463. The MS^2 spectrum revealed the loss of a hexoside residue (162 Da) giving the aglycone ion, Y_0^-, at *m/z* 301 as base peak.

Its MS^n fragmentation led to the detection of RDA fragment ions at *m/z* 179 ([$^{1,2}A^-$−H]$^-$), 271 ([M−H−CH_2O]$^-$), 255 ([M−H−H_2O−CO]$^-$) and 151 ($^{1,2}A^-$−CO) which are characteristic ions of quercetin (Gouveia & Castilho, 2009, 2010). Compound **14** was identified by comparison of MS^n and UV data (bands at 258 nm and 353 nm) obtained for a standard solution of quercetin-3-*O*-glucoside.

This compound was only found in the leaves of *H. obconicum*, and it has been already reported in *Helichrysum devium* and *Helichrysum melaleucum* (Gouveia & Castilho, 2009, 2010).

Compound **47** (t_R = 34.2 min) showed a [M−H]$^-$ ion at *m/z* 269. Comparison of the main MS^n fragment ions such as those at *m/z* 225 ([M−H−CO_2]$^-$), 201 ([M−H−C_3O_2]$^-$), 151 ($^{1,3}A$) and 149 ($^{1,4}B$ + 2H), UV spectrum (bands at 250 and 332 nm) and the HPLC retention time to those obtained for a standard solution allowed for the identification of compound **47** as apigenin. It was only

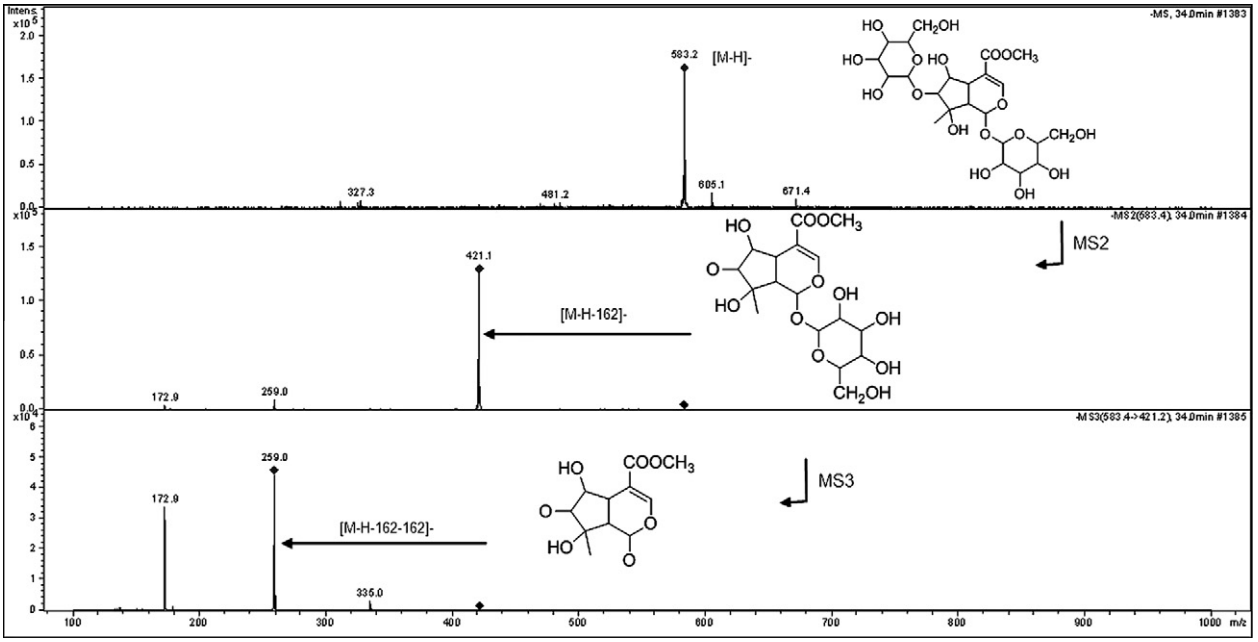


Fig. 4. ESI-MS^n negative mode of compound **46** – lamiridosins-di-*O*-hexoside. Sequential fragmentation, MS^n (n = 2–3), of the ion at *m/z* 583.

found in the leaves extract, although all extracts gave a substituted form of apigenin (compound **24**).

Compound **24** (t_R = 15.7 min) gave a $[M-H]^-$ ion at *m/z* 445 and subsequent fragmentation showed the loss of 176 Da, which corresponds to a glucuronic acid moiety. Fragmentation of the aglycone ion, Y_0^-, at *m/z* 269 gave fragment ions at *m/z* 227, 225, 169, 151 and 149. This fragmentation behaviour is similar to that found for apigenin (compound **47**). As it is known, for flavones, the most common substitution position is the 7-OH, therefore compound **24** was identified as apigenin-7-*O*-glucuronide. All morphological parts analysed of *H. obconicum* revealed the presence of this compound.

Compound **46** (t_R = 34.0 min) exhibited a $[M-H]^-$ ion at *m/z* 583 and its MS^2 spectrum gave a fragment ion at *m/z* 421 due to the loss of 162 Da probably a hexoside residue. MS^3 fragmentation of the ion at *m/z* 421 showed the loss of a 162 Da residue forming a fragment ion at *m/z* 259 assigned as the aglycone ion, Y_0^-. The aglycone ion under MS^4 fragmentation displayed a fragment ion at *m/z* 173 (Fig. 4). Comparing these results with literature data, the ion at *m/z* 421 was identified as lamalbid (Alipieva, Kokubun, Taskova, Evstatieva, & Handjieva, 2007). Lamalbid belongs to the group of iridoids and is a hexoside of lamiridosins. Lamiridosins (aglycone part) is the name given to the two inseparable epimers (carbon 1 is a chiral carbon) (Zhang et al., 2009). Therefore, compound **46** was identified as lamiridosins-di-*O*-hexoside (Fig. 4).

This class of compounds consists in a large group of natural monoterpenes with taxonomic marker properties. More precisely, lamiridosins are known for its anti-HCV *pp* activity (Zhang et al., 2009).

3.3. Unidentified compounds

Two compounds with $[M-H]^-$ ion at *m/z* 497 were observed at a retention time of 23.4 and 24.1 min. However, their MS^n fragmentation behaviour was very distinct to that found for compounds **33** and **35**, also with $[M-H]^-$ ion at *m/z* 497.

MS^2 fragmentation showed the loss of 224 Da to form a fragment ion at *m/z* 273. Further MS^n fragmentation gave fragment ions at *m/z* 109 and 168. However, it was not possible to positively identify them.

Compound **44** (t_R = 33.0 min) showed a $[M-H]^-$ ion at *m/z* 707 and in the MS^3 spectrum the loss of 162 Da residue was observed, probably due to a hexoside residue. It was only found in the leaves extract.

Compound **49** (t_R = 37.7 min) showed a $[M-H]^-$ ion at *m/z* 287 and its MS^2 fragmentation gave a fragment ion at *m/z* 269 (loss of 18 Da, probably a H_2O molecule). The ion at *m/z* 269 is characteristic for apigenin (compound **47**). However, the low intensity of this ion did not allowed for further fragmentation in order to fully characterise it and the UV spectrum did not provide any valid information; thus identification of compound **49**, only found in the stems, was not achieved.

4. Conclusion

50 phenolic compounds were characterised in the different morphological parts of *H. obconicum*, based on their HPLC retention time, UV spectra and mass fragmentation behaviour.

Dicaffeoylquinic acids and specifically malonyl-dicaffeoylquinic acids are the main components of *H. obconicum* methanolic extracts. Other derivatives of the esters of caffeic acid and quinic acid or shikimic acids were also detected in minor amounts. Moreover, it was the first time, to our knowledge, that shikimic acid derivatives and iridoids hexosides were found in *Helichrysum* species.

The phenolic composition of *H. obconicum* shared 20 of its 50 components with *H. devium* or *H. melaleucum* albeit in very different proportions. This conclusion is in good agreement with the fact that *H. obconicum* is used in the local traditional medicine with different purposes (stomachic diseases) of the two other endemic species, used for respiratory problems.

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