

# *Helichrysum monizii* Lowe: Phenolic Composition and Antioxidant Potential

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## ABSTRACT:

**Introduction** – In Madeira Archipelago there are four endemic *Helichrysum* species and three of them are used in the traditional medicine. *Helichrysum monizii* is a rare endemism with very scarce information available concerning its uses in the local traditional medicine.

**Objective** – The aim of this work was to study for the first time *Helichrysum monizii* in terms of its antioxidant capacity and the identification of the phenolic compounds to which that activity is due.

**Methodology** – Three different methods of extraction were performed and total phenolic and flavonoid contents of extracts were correlated to radical scavenging and antioxidant capacity by DPPH, ABTS, FRAP and  $\beta$ -carotene assays. An HPLC-DAD-ESI/MS<sup>n</sup> method was employed for the separation and identification of the phenolic and flavonoid components. **Results** – The results revealed a high antioxidant potential mainly related to the phenolic profile of the plant. Polar components of methanol extracts of *Helichrysum monizii* were detected by a high-performance liquid chromatography/electrospray ionisation tandem mass spectrometry (HPLC-DAD-ESI/MS<sup>n</sup>) method. Thirty-three compounds were identified and 19 of them were identified as quinic acid derivatives.

**Conclusions** – The high antioxidant potential *Helichrysum monizii* was for the first time established. Dicafeoylquinic acids are the main responsible for that activity. Copyright © 2011 John Wiley & Sons, Ltd.

**Keywords:** antioxidant; electrospray; flavonoids; *Helichrysum*; phenolics

## Introduction

The *Helichrysum* Mill. (family of *Asteraceae*) genus includes more than 500 species that are widespread around the world. A great number of biological activities are usually attributed to this genus, such as anti-inflammatory, anti-allergic, antioxidant, antimicrobial, cough relief and treatment of cold and wounds (Albayrak *et al.*, 2010).

This genus is represented in Madeira Archipelago (Portugal) by several imported species and by four endemic ones used in folk medicine as well as in gardening as decorative shrubs. These four endemic species are: *Helichrysum devium* Johns., *Helichrysum melaleucum* Rchb. Ex Holl, *Helichrysum obconicum* DC. and *Helichrysum monizii* Lowe. Our recent work has been focused on the study of these endemic species. The three first plants mentioned above are well known for their use in traditional medicine with different purposes (Gouveia and Castilho, 2009, 2010). The study of the endemic population of *Helichrysum* subspecies would not be complete without *Helichrysum monizii* Lowe, nowadays an extremely rare plant that inhabits only a few cliffs on south coast of Madeira. According to expert botanists, only about six specimens survive in the wild, in locations that are very difficult to access. To overcome the threat of extinction, Madeira Botanical Garden recently developed a programme for greenhouse reproduction of this plant and has kindly provided us with a small sample of plant material for this investigation.

Owing to its scarcity, *Helichrysum monizii* is not considered a useful plant and there is no scientific research in terms of its biological activity and its phenolic composition. The investigation of these points comprises the main novelty and interest of this work. Plant phenolic compounds are plant secondary metabolites. They possess the capacity to scavenge free radicals and can

interact with biological systems in order to prevent diseases associated with lipid oxidation (Katalinic *et al.*, 2010). The consumption of antioxidants is believed to prevent or reduce the risk of lipid oxidation associated with a high number of major health problems and also food deterioration. The search for natural sources of antioxidants with low cytotoxicity has increased dramatically over the years. Therefore, the aims of this work were to evaluate the total phenolic and flavonoid content, to evaluate the antioxidant capacity of the methanolic extracts and to establish its phenolic composition by HPLC-DAD-ESI/MS<sup>n</sup>, in order to compare this plant with the other endemic subspecies.

## Experimental

### Chemicals and standards

The following reagents were purchased from Merck (Darmstadt, Germany): potassium persulphate (99%), sodium chloride (99.5%), disodium phosphate dodecahydrated (99%), glacial acetic acid (100%), sodium carbonate (p.a.) and ferrous sulphate heptahydrate (99%). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (> 95%), Trolox ( $\geq$  99.8%, HPLC), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) ( $\geq$  99%, HPLC), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) ( $\geq$  99.0%, TLC),  $\beta$ -carotene ( $\geq$  97%, UV), Tween 40 and Folin-Ciocalteu's phenol reagent were purchased from

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Fluka (Lisbon, Portugal). Potassium chloride (> 99.5%), gallic acid (99%, HPLC), potassium acetate (p.a.), rutin ( $\geq$  98%, HPLC) and ferric chloride hexahydrate (97–100%) were purchased from Panreac (Barcelona, Spain); potassium dihydrogen phosphate (99.5%), aluminium chloride (98%) and sodium acetate trihydrate (pure) were purchased from Riedel-de Haën (Hanover, Germany).

All solvents used for plant extraction were AR-grade, purchased from Fisher (Lisbon, Portugal). HPLC-MS-grade acetonitrile (99.9%, LabScan, Gliwice, Poland) and ultra-pure water (Milli-Q Waters purification system, EUA) were used for HPLC analysis.

Stock solutions of standard compounds (100 µg/mL) were prepared in ethanol for HPLC-DAD-ESI/MS<sup>n</sup> identification and stored in a refrigerator at –20°C until use. Standards used were: *p*-coumaric acid (> 99%), caffeic acid (> 99%), protocatechuic acid (> 99%) from Extrasynthese (Lyon, France), and 5-*O*-caffeoylquinic acid (99%) from Acros Organics (Geel, Belgium); 1,3-*O*-dicafeoylquinic acid, 1,5-*O*-dicafeoylquinic acid, 3,4-*O*-dicafeoylquinic acid, 3,5-*O*-dicafeoylquinic acid, 4,5-*O*-dicafeoylquinic acid and 3,4,5-*O*-tricafeoylquinic acid (> 98% by HPLC for all) were obtained from Chengdo Biopurify Phytochemicals Ltd China (Sichuan, China).

### Plant material and sample preparation

A sample of *Helichrysum monizii* was obtained from Madeira Botanical Garden collection. The aerial parts were dried (at room temperature and protected from direct sunlight) and ground to fine powder in a mechanic grinder. Then the powdered plant material was extracted by three different methods:

- (1) ultrasound-assisted solvent extraction – plant material (1 g) was extracted with methanol (25 mL) using a sonicator Bandelin Sonorex (Germany) at 35 kHz and 200 W for 60 min at room temperature.
- (2) solid–liquid extraction – plant material (1 g) was extracted with methanol (25 mL) at room temperature for 24 h.
- (3) Soxhlet extraction – plant material (3 g) was extracted using a Soxhlet-type extractor with methanol (200 mL) at 60°C for 4 h.

In all cases the solutions were filtered and concentrated under reduced pressure in a rotary evaporator (40°C) and kept in the dark at 4°C until tested.

### Total phenolic content

The total content of phenolic compounds of the extracts was determined following the Folin–Ciocalteu method (Zheng and Wang, 2001) with some modifications. Plant extracts were dissolved in methanol to yield a concentration (w/v) 10 mg/mL. Each sample solution (50 µL) was mixed with 1.25 mL of Folin–Ciocalteu reagent (diluted 1:10 fold) and 1 mL of 7.5% sodium carbonate solution. The mixture was incubated for 30 min at room temperature and then absorbance was measured at 765 nm. The final results were expressed as gallic acid equivalents per 100 gram of dried plant (mg GAE/100 g dried plant).

### Total flavonoid content

Total flavonoid content was measured using a modified method (Akkol *et al.*, 2008). Briefly, 10 mg of extract was dissolved in 5 mL of methanol. In a 10 mL test tube, 0.5 mL of sample solution, 1.5 mL of methanol, 2.8 mL of water, 0.1 mL of potassium acetate (1 M) and 0.1 mL of aluminium chloride (10% in methanol) were mixed. The decrease in absorbance was measured at 415 nm after incubation at room temperature for 30 min. The total flavonoid content was expressed as milligrams of rutin equivalent per 100 gram of dried sample (mg RUE/100 g dried plant).

### ABTS<sup>•+</sup> radical scavenging activity

The method of decolourisation of free radical ABTS<sup>•+</sup> used was a modified version of that initially reported by Re *et al.* (1999). The ABTS<sup>•+</sup>

radical was prepared by reacting 50 mL of 2 mM ABTS<sup>•+</sup> solution with 200 µL of 70 mM potassium persulphate solution. This mixture was kept in the dark for 16 h at room temperature. This solution was stable in this form for two days (Re *et al.*, 1999). For each analysis the ABTS<sup>•+</sup> solution was diluted with pH 7.4 phosphate buffered saline (PBS) solution to an initial absorbance of  $0.700 \pm 0.021$  at 734 nm. This solution was freshly prepared for each analysis. For the assessment of the radical scavenging activity, an aliquot of 100 µL methanolic solution with a concentration (w/v) of 10 mg/mL was added to 1.8 mL of ABTS<sup>•+</sup> solution and the absorbance decrease, at a 734 nm, was recorded during 6 min. Results were expressed in terms of micromoles Trolox equivalent per 100 grams of dried plant antioxidant capacity (µmol equiv. Trolox/100 g dried plant).

### DPPH radical scavenging activity

The DPPH method was executed according to the method of Gordon *et al.* (2001), introducing some modifications. Briefly, 100 µL of the methanolic solutions (10 mg/mL) were added to 3.5 mL of a 0.06 mM methanol DPPH radical solution. The decrease in absorbance at 516 nm was measured every minute during 30 min, in the dark. The DPPH radical scavenging effect of the extracts was expressed, based on the Trolox calibration curve as µmol Trolox equivalent per 100 gram of dried plant (µmol equiv. Trolox/100 g dried plant).

### Ferric reducing activity

The ferric reducing ability of the extracts was assessed based on the ferric reducing activity (FRAP assay) assay (Benzie and Strain, 1996). FRAP reagent was prepared daily by mixing 2.5 mL of solution ferric trichloride hexahydrate (20 mM), 2.5 mL of solution TPTZ (10 mM in 40 mM of hydrochloric acid) and 25 mL of acetate buffer 0.3 M (pH 3.6) and incubating at 37°C. The extracts were dissolved in methanol to yield a final concentration of 1 mg/mL. For each analysis, 30 µL of methanolic solution was added to 180 µL of distilled water and 1.8 mL of FRAP solution. The increase in absorbance was recorded at 593 nm in 15 s intervals, during 30 min at 37°C. The FRAP results were expressed as mmol Fe(II) sulphate heptahydrate per mg of dried plant [mmol Fe(II)/mg dried plant].

### β-Carotene bleaching assay

The method described in literature (Siddhuraju and Becker, 2003) was applied with some modifications. Briefly, 2 mL of β-carotene solution 0.2 mg/mL in chloroform was added to a round-bottom flask, containing 0.04 mL of linoleic acid and 200 mg of Tween 40. The chloroform was removed by evaporation using nitrogen and then 50 mL of oxygenated ultrapure water, obtained by bubbling air through the water for 15 min, was added. The mixture was vigorously shaken. The resulting emulsion was freshly prepared before each experiment. Stock solutions of the extracts were prepared in ethanol to yield a final concentration of 1 mg/mL. An aliquot of 250 µL of the β-carotene–linoleic acid emulsion was distributed in each of the wells of the 96-well microtitre plates and 30 µL of the samples solutions were added. An equal amount of ethanol was used as control. The samples were then subjected to thermal autoxidation at 45°C for 210 min. The solution's absorbance was measured at 490 nm, at 15 min intervals. The antioxidant activity (AA) of each sample was evaluated in terms of the bleaching of β-carotene using the following equation:  $AA (\%) = (1 - A_0 - A_t/A'_0 - A'_t) \times 100$ , where  $A_0$  and  $A'_0$  are the absorbance values measured at zero incubation time for the test and control respectively and  $A_t$  and  $A'_t$  are the corresponding absorbance values measured after incubation for 210 min.

### HPLC-DAD-ESI/MS<sup>n</sup> analysis

**Liquid chromatography.** Stock solutions with concentrations (w/v) of 5 mg/mL were prepared by dissolving each dried extract in initial HPLC mobile phase (acetonitrile:water, 20:80, v/v). These solutions were

filtered through 0.45 mm Nylon micropore membranes prior to use and 10 mL were injected for HPLC-DAD-ESI/MS<sup>n</sup> analysis. Three independent assays were performed for each sample.

The HPLC analysis was performed on a Dionex ultimate 3000 series instrument (California, EUA) coupled to a binary pump, a diode-array detector (DAD), an autosampler and a column compartment. Samples were separated on a Phenomenex Gemini C<sub>18</sub> column (5 µm, 250 × 3.0 mm i.d.) 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 programme 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 350 nm and spectral data for all peaks were accumulated in the range of 190–400 nm. Column temperature was controlled at 30°C.

**Mass spectrometry.** For HPLC-ESI/MS<sup>n</sup> 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 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<sub>2</sub>) was used both as drying gas at a flow 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. Ultra-high-purity helium (He) was used as collision gas at a pressure of  $1 \times 10^{-5}$  mbar and the collision energy was set at 40 V.

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

### Statistical analysis

All measurements were performed in triplicates and results are expressed as mean ± SD. Significant differences in antioxidant activity, total phenolic and flavonoid content of the different extracts were determined using one-way ANOVA. The statistical probability was considered to be significantly different at the level of  $p < 0.05$ .

## Results and Discussion

### Extraction yield, total phenolic content and total flavonoid content

Three different methods of extraction, based on liquid–solid extractions, were used to obtain methanolic extracts of *Helichrysum monizii*. The yield percentages obtained for each of the procedures are shown in Table 1. The values were 3.56% (maceration), 12.49% (ultrasonication) and 16.0% (Soxhlet extraction). Soxhlet extraction showed a higher, but not remarkably higher, yield (ca. 4% higher) than the ultrasonication extraction, considering that this last one uses a smaller amount of sample, volume of solvent and time of extraction. Soxhlet extraction has the disadvantage of possible deterioration of thermolabile compounds (Kaufman *et al.*, 1999). Nevertheless, we compared the phenolic and flavonoid composition and evaluated the antioxidant capacity of extracts from *Helichrysum monizii* obtained by the three methods. The extract obtained by the Soxhlet method showed the higher phenolic content at  $369.35 \pm 8.15$  mg GAE/100 g, followed by the ultrasonication and maceration extraction gave the lowest phenolic content. The total flavonoid content revealed the same relative order: maceration ( $4.69 \pm 0.0139$  mg RUE/100 g) < ultrasonication extraction ( $17.31 \pm 0.52$  mg RUE/100 g) < Soxhlet ( $19.50 \pm 0.70$  mg RUE/100 g).

These orders of values, found in both types of compounds, are related to the capacity of the extraction method to release these substances. Soxhlet extraction provided the highest total phenolic and flavonoid contents, which can be explained by the fact that higher temperatures of extraction affect the integrity of the cell wall and therefore induce a higher recovery of metabolites (Li *et al.*, 2006). However, as the HPLC-MS<sup>n</sup> methods of analysis demonstrated, some of the components are degraded by high temperature and some artefacts are present in the chromatogram. Maceration, as expected, was proven to be the less effective method of extraction.

### Antioxidant assays

The antioxidant properties of natural products, such as plant extracts, are due to the nature of the bioactive compounds and sometimes to synergistic effects between them. However, the establishment of the contribution of each individual component to the total antioxidant activity is difficult and time consuming. The common procedure is to measure the total antioxidant capacity of the whole sample.

There are several methodologies widely used and, in this work, we determined the free radical scavenging capacity of *Helichrysum monizii* using ABTS and DPPH methods, and their reducing capacity by the FRAP method and the β-carotene bleaching assay. The use of several methods to measure antioxidant activity may seem a redundancy, but since different authors used various methods, comparison of properties becomes easier if a large set of data is available.

### ABTS<sup>+</sup> radical scavenging activity

The cation radical ABTS<sup>+</sup> consumption due to the *Helichrysum monizii* samples was measured using the calibration curve for Trolox (8–270 µM,  $R^2 = 0.9995$ ). The ABTS values of *Helichrysum monizii* samples extracted by different methods are presented in Table 1. All extracts showed the capacity to neutralise the radical cation ABTS<sup>+</sup> but at different levels significantly different at  $p < 0.05$ . The lowest activity was obtained from the maceration extraction with a value of  $3167.73 \pm 1.42$  µmol equiv. Trolox/100 g, followed by the ultrasonication and Soxhlet extraction with values of  $10856.12 \pm 9.44$  and  $25023.93 \pm 79.93$  µmol equiv. Trolox/100 g, respectively.

### DPPH radical scavenging activity

The purple solution of the free radical DPPH gives a characteristic absorption at 516 nm which decreases by reaction with samples rich in electron or hydrogen donors. The slower the decrease in absorbance, at the same concentration, the higher the antioxidant capacity of the extract.

A large range of activity was obtained by the DPPH method with values varying from  $9917.63 \pm 33.63$  µmol equiv. Trolox/100 g (maceration) to  $46176.43 \pm 3.78$  µmol equiv. Trolox/100 g (Soxhlet extraction). The same order of activity observed in the ABTS method was found in the DPPH method, confirmed by the very good correlation ( $R^2 = 1$ ) between these two methods. Still, the ABTS values are lower than those obtained from the DPPH assay. This can probably be attributed to the fact that the ABTS<sup>+</sup> solution is more easily decolourised by compounds with high molecular weights such as tannins (Siddhuraju, 2007). Nevertheless, the ABTS assay has the advantage of the working solution being soluble in

**Table 1.** *Helichrysum monizii* experimental determinations of total phenolic and flavonoid content and antioxidant capacity against ABTS, DPPH, FRAP and  $\beta$ -carotene

<i>Helichrysum monizii</i>	Methanolic extraction		
	Maceration	Ultrasonication	Soxhlet
Extraction yield (%)	3.56	12.49	16.00
Total phenolic content (mg GAE/100 g dried plant)	131.341 $\pm$ 3.32	170.948 $\pm$ 5.94	369.346 $\pm$ 8.15
Total flavonoids content (mg RUE/100 g dried plant)	4.686 $\pm$ 0.0138	17.312 $\pm$ 0.516	19.489 $\pm$ 0.70
ABTS ( $\mu$ mol equiv. Trolox/100 g dried plant)	3167.73 $\pm$ 1.42	10856.12 $\pm$ 9.44	25023.74 $\pm$ 79.93
DPPH ( $\mu$ mol equiv. Trolox/100 g dried plant)	9917.63 $\pm$ 33.63	22576.88 $\pm$ 54.14	46176.43 $\pm$ 31.78
FRAP [mmol Fe(II)/mg dried plant]	24352.0 $\pm$ 35.2	35854.4 $\pm$ 64.3	38014.5 $\pm$ 396.9
$\beta$ -Carotene (%) (1 mg/mL)	3.87 $\pm$ 1.31	5.36 $\pm$ 0.08	7.86 $\pm$ 0.41

aqueous and organic solvents, at a large range of pH values, and the reaction time is lower than the DPPH assay. The benefit of employing the DPPH assay is due to the higher stability of the DPPH radical and its commercial form being ready to use.

### FRAP assay

The FRAP assay measures the antioxidant properties of any sample based on its reducing ability. It is a method based on the colour change where the yellow ferric-tripyridyltriazine complex is reduced to the blue ferrous complex. This reaction is pH-dependent (ideal pH 3.6). The FRAP results are expressed as mmol FeSO<sub>4</sub> equivalents per mg of dried extract and the results show that the Soxhlet extraction, once more, provided the extract with higher antioxidant capacity with a FRAP value of 38014.5  $\pm$  396.9 mmol Fe (II)/mg. The values obtained for the other extracts (Table 1) are significantly different ( $p < 0.05$ ), but not as different as observed in the ABTS and DPPH assays.

Based on these results, it is possible to infer that the three types of extraction studied are effective in the recuperation of compounds with good reducing capacity and good electron donors. The FRAP values showed a reasonable correlation with the ABTS and DPPH data, meaning that compounds present in the samples with radical scavenge capacity also have some reducing capacity.

### $\beta$ -Carotene bleaching assay

In this assay, the peroxy radicals formed when linoleic acid is oxidised attack the highly unsaturated  $\beta$ -carotene molecules that consequently undergo fast decolourisation (Katalinic *et al.*, 2010). The presence of antioxidants in the sample will reduce the oxidation of  $\beta$ -carotene. According to some authors, this process reaches a plateau phase after 90–120 min of reaction time (Parejo *et al.*, 2002). However, for *Helichrysum monizii* samples a reaction time of 210 min was necessary.

Several concentrations (10–1 mg/mL) were studied for each extract. None of the samples completely inhibited the  $\beta$ -carotene oxidation and the most powerful extract was that obtained from the Soxhlet extraction. For a concentration of 10 mg/mL the inhibition of  $\beta$ -carotene was similar to all samples, despite of being statistically different (Table 1). For the lowest concentration (1 mg/mL), the Soxhlet extract presented a higher antioxidant capacity (7.86  $\pm$  0.41%) followed by the ultrasonication extraction (5.36  $\pm$  0.08%) and maceration (3.87  $\pm$  1.31%). These data revealed that the extract obtained by Soxhlet extraction has a composition

with higher capacity to prevent the oxidation of lipids and can be used as preservatives to delay or limit lipids oxidation.

The ABTS and DPPH results were poorly correlated with the  $\beta$ -carotene values, probably due to a weak solubility in the two-phase medium of  $\beta$ -carotene reaction of the most active compounds against ABTS and DPPH radicals.

### Correlations between the total phenolic and flavonoid content and antioxidant assays

It is well known that the antioxidant activity of a plant extract largely depends on both the composition of the extract and the test system. The antioxidant activity can be influenced by a large number of factors, and cannot be fully evaluated by one single method due to the various mechanisms of antioxidant action. Table 2 shows the correlations (linear regression coefficients,  $R^2$ ) between the total flavonoid contents (TFC), total phenolic contents (TPC) and the four antioxidant assays results for *Helichrysum monizii* samples. The radical scavenging methods (ABTS and DPPH) showed a satisfactory correlation with the TFC ( $R^2$  ca. 0.72) and a very good correlation ( $R^2$  ca. 0.96) with the TPC, indicating that most phenolic compounds extracted from *Helichrysum monizii* were capable of reacting in both methods.

The antioxidant capacity of phenolic acid derivatives is normally dependent on the number of hydroxyl and methoxyl groups linked to the aromatic ring. For example, polyphenols are more effective than the monophenols, and cinnamic acid derivatives display higher antioxidant capacity than their benzoic counterparts (Kim and Lee, 2004). The radical scavenging capacity of flavonoids is structure-dependent (Silva *et al.*, 2002), the presence of a catechol group in the B-ring being essential for a good scavenging activity. The conjugation of the B-ring is also important to ensure electron-delocalisation. This

**Table 2.** Correlations values ( $R^2$ ) between the antioxidant assays results and total flavonoid content (TFC) and total phenolic content (TPC) of *Helichrysum monizii* samples

$R^2$	TFC	TPC
ABTS	0.7184	0.9624
DPPH	0.7210	0.9612
FRAP	0.9999	0.5413
$\beta$ -Carotene	0.8891	0.7105



structure–activity dependency can be the explanation for the observed correlation decrease observed.

The opposite was found with the FRAP assay (Table 2). FRAP results were extremely well correlated ( $R^2=0.9999$ ) to the total flavonoid content but poorly with the total phenolic content ( $R^2=0.5413$ ). This result was somehow unexpected since the Folin–Ciocalteu method used for TPC determination is based on the reducing properties of the sample. However, the correlation between antioxidant assays and TPC is usually established for TPC values obtained from different samples obtained by the same extraction method; therefore variations are quantitative rather than qualitative. In the present work, the TFC and TPC values we are plotting against FRAP results were obtained from different methods of extraction, affecting different types of compounds: for instance, Soxhlet extraction yielded 4% more extract than ultrasonication but the TPC in it is more than double, while the TFC increased in only 2% (Table 1). The Folin–Ciocalteu assay is not specific for particular groups of phenolic compounds and is affected by interfering substances such as organic acid, sugars and organic non-phenolic acids (Singleton *et al.*, 1999). It may happen that these substances are good radical scavengers but poor reducing agents, thus leading to nonlinear results.

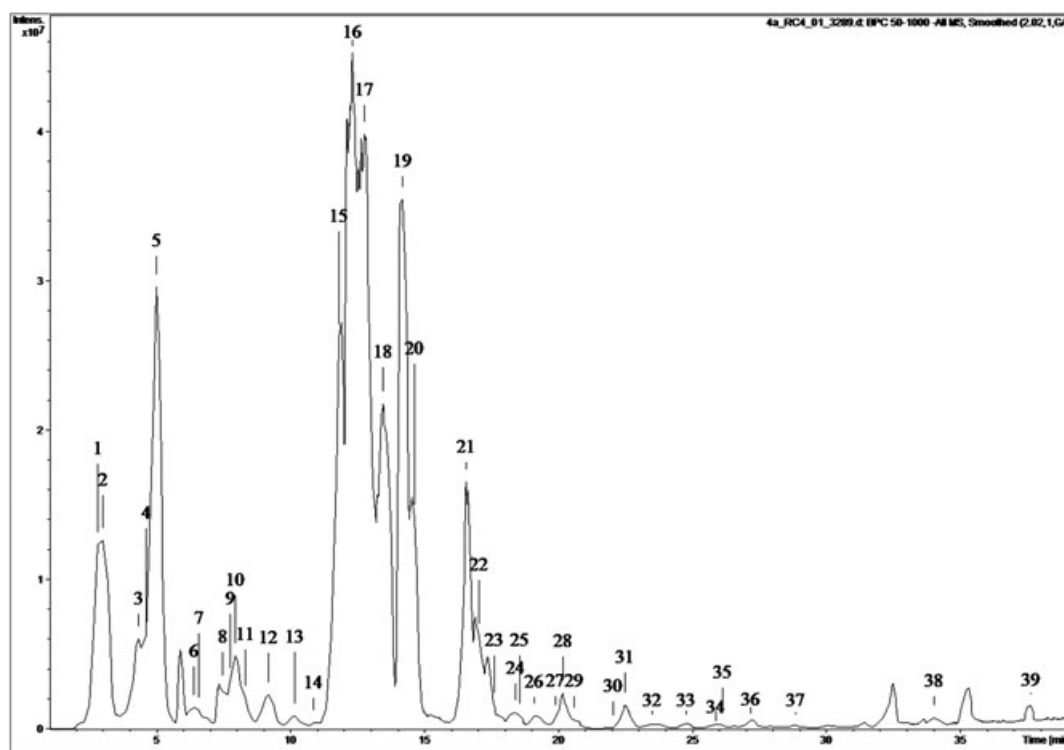
The most relevant observation concerning the antioxidant properties of *H. monizii* is that its methanol extract is at least 10 times more active, in all performed assays, than those of the other three *Helichrysum* subspecies we have studied using exactly in the same conditions (data to be published elsewhere). Also, in a study of 112 species (Cai *et al.*, 2004) only six showed higher activity on the ABTS assay, and those were aqueous extracts rich in flavonols and tannins, considered to have higher individual activity than hydroxycinnamic acids.

### HPLC-DAD-ESI/MS<sup>n</sup> analysis

Considering the high antioxidant capacity of the methanolic extracts from *Helichrysum monizii*, its phenolic composition was investigated by a HPLC-DAD-ESI/MS<sup>n</sup> method. The optimised HPLC conditions were obtained after evaluating several parameters, such as mobile phase (composition and pH), sample concentration, volume of injection and ionisation parameters. The conditions that showed the best separation and resolution were those described above.

Despite Soxhlet extract exhibiting the highest antioxidant capacity in the *in vitro* antioxidant assays, the HPLC-DAD-ESI/MS<sup>n</sup> screening of the extract obtained by ultrasonication displayed a chromatogram with better resolution and fragmentation patterns. As mentioned before, ultrasonication was an efficient method for extracting active components (which extraction by maceration was not capable to do) without the risk of thermal degradation associated with Soxhlet extraction. Thus, the ultrasonication extract was selected to run further analysis for separation and identification of the phenolic composition of *Helichrysum monizii*.

Thirty-seven peaks were detected and 33 were characterised. Most of them showed typical hydroxycinnamic acid UV absorptions ( $\lambda_{\max}$  230–240, 300 sh. 320–340 nm) suggesting that the large percentage of the components are hydroxycinnamic conjugates (Mabry *et al.*, 1970). Seven components were undoubtedly identified by comparison with commercially available standards: characterisation of the peaks was achieved by comparison of the HPLC retention time, UV and mass spectra of standard solutions. Since there are no standard compounds for the majority of the peaks, the proposed structure of the other compounds was based mainly on the MS<sup>n</sup> fragmentation mechanisms and complemented



**Figure 1.** HPLC-DAD-ESI/MS<sup>n</sup> analysis of the methanolic extract of *Helichrysum monizii* total aerial parts; LC-MS negative ion ESI-MS base peak chromatogram.

**Table 3.** Characterisation of phenolic compounds of the methanolic extract of total aerial parts from *Helichrysum monizii* by HPLC-DAD-ESI/MS<sup>n</sup>

No.	t <sub>R</sub> (min)	UV λ <sub>max</sub> (nm)	[M – H] <sup>–</sup> m/z	HPLC-DAD-ESI/MS <sup>n</sup> m/z (% base peak)	Assigned identity
1	2.9	294, 324	683	MS <sup>2</sup> [683]: 341 (100) MS <sup>3</sup> [683 → 341]: 179 (100), 161 (23.0), 119 (28.4), 113 (28.7) MS <sup>4</sup> [683 → 341 → 179]: 161 (100), 135 (36.3), 113 (44.1), 89 (49.0)	Caffeic acid-O-hexoside
2	3.1	—	191	MS <sup>2</sup> [191]: 173 (46.2), 171 (35.8), 127 (100), 109 (23.3), 93 (38.9), 85 (56.3) MS <sup>3</sup> [191 → 127]: 109 (100), 85.3 (49.4)	Quinic acid
3	4.3	241, 298, 324	353	MS <sup>2</sup> [353]: 191 (100), 179 (60.6), 135 (18.) MS <sup>3</sup> [353 → 191]: 173 (49.3), 127 (100), 111 (15.1), 87 (20.5), 85 (17.8) MS <sup>4</sup> [353 → 191 → 127]: 109 (100), 85 (64.6)	3-O-Caffeoylquinic acid
4	4.6	—	317	MS <sup>2</sup> [317]: 315 (16.8), 225 (100), 165 (21.5), MS <sup>3</sup> [317 → 225]: 207 (62.2), 165 (84.0), 127 (51.4), 125 (100) MS <sup>4</sup> [317 → 225 → 125]: 79 (100)	Unknown
5 <sup>a</sup>	5.0	242, 300, 325	707	MS <sup>2</sup> [707]: 354 (10.8), 353 (100) MS <sup>3</sup> [707 → 353]: 191 (100) MS <sup>4</sup> [707 → 353 → 191]: 173 (41.8), 127 (100), 111 (22.7), 85 (20.3)	5-O-Caffeoylquinic acid
6 <sup>a</sup>	6.4	243, 300, 321	515	MS <sup>2</sup> [515]: 353 (100), 335 (28.4), 191 (12.8), 179 (34.5) MS <sup>3</sup> [515 → 353]: 191 (100), 179 (58.5), 135 (22.9) MS <sup>4</sup> [515 → 353 → 191]: 127 (90.1), 111 (58.7), 109 (100)	1,3-O-Dicaffeoylquinic acid
7	7.0	210, 304	337	MS <sup>2</sup> [337]: 192 (11.8), 191 (100) MS <sup>3</sup> [337 → 191]: 173 (39.1), 171 (50.8), 127 (100), 85 (17.5), 81 (26.4)	cis 5-O-p-Coumaroylquinic acid
8	7.4	327	367	MS <sup>2</sup> [367]: 191 (100), 173 (5.6) MS <sup>3</sup> [367 → 191]: 173 (34.4), 111 (40.5), 127 (81.7), 85 (100)	5-O-Feruloylquinic acid
9	7.5	291, 320	533	MS <sup>2</sup> [533]: 372 (18.5), 371 (100), 353 (13.3) MS <sup>3</sup> [533 → 371]: 353 (100), 191 (22.3), 173 (47.7), 135 (64.9)	Caffeic acid derivative
10	7.9	212, 305	675	MS <sup>2</sup> [675]: 338 (11.1), 337 (100) MS <sup>3</sup> [675 → 337]: 191 (100) MS <sup>4</sup> [675 → 337 → 191]: 173 (44.7), 127 (100), 109 (51.1), 93 (16.7), 85 (82.5)	5-O-p-Coumaroylquinic acid
11	8.3	—	677	MS <sup>2</sup> [677]: 516 (18.0), 515 (100) MS <sup>3</sup> [677 → 515]: 353 (34.0), 341 (26.1), 323 (100), 191 (48.1), 179 (15.3) MS <sup>4</sup> [677 → 515 → 323]: 161 (100), 133 (15.6)	Dicaffeoylquinic acid hexoside
12	9.1	—	547	MS <sup>2</sup> [547]: 312 (14.7), 311 (100), 293 (25.1), 161 (10.2) MS <sup>3</sup> [547 → 311]: 293 (100), 221 (39.1), 191 (46.5), 161 (33.8), 149 (10.7) MS <sup>4</sup> [547 → 311 → 293]: 207 (75.8), 149 (100), 131 (54.3), 87 (51.9)	Unknown
13	10.0	254, 272, 340	477	MS <sup>2</sup> [477]: 316 (17.0), 315 (100), 300 (12.7) MS <sup>3</sup> [477 → 315]: 301 (25.5), 300 (100) MS <sup>4</sup> [477 → 315 → 300]: 272 (47.9), 255 (100), 216 (90.0), 215 (57.2), 211 (78.1)	Isorhamnetin-O-hexoside
14	10.8	—	549	MS <sup>2</sup> [549]: 388 (45.8), 387 (100) MS <sup>3</sup> [549 → 387]: 372 (49.4), 181 (60.2), 166 (77.1), 151 (100) MS <sup>4</sup> [549 → 387 → 151]: 136 (100)	Medioresinol-O-hexoside
15 <sup>a</sup>	12.0	246, 300, 323	515	MS <sup>2</sup> [515]: 353 (100), 335 (11.8), 191 (10.2), 179 (16.9), 173 (38.1) MS <sup>3</sup> [515 → 353]: 191 (43.1), 179 (69.7),	3,4-O-Dicaffeoylquinic acid

**Table 3.** (Continued)

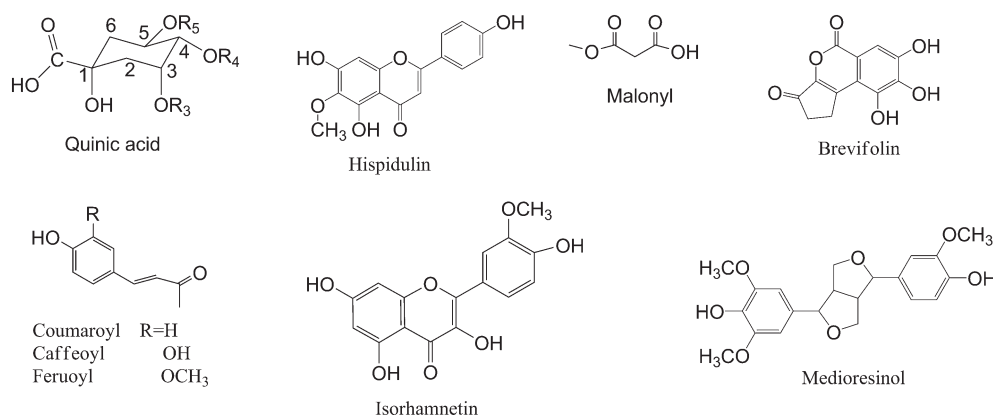
No.	$t_R$ (min)	UV $\lambda_{max}$ (nm)	$[M-H]^-$ $m/z$	HPLC-DAD-ESI/MS <sup>n</sup> $m/z$ (% base peak)	Assigned identity
16 <sup>a</sup>	12.5	243, 300, 328	515	173 (100), 135 (20.8) MS <sup>4</sup> [515 → 353 → 173]: 155 (57.1), 137 (38.8), 127 (33.3), 111 (100), 93 (85.6), 83 (37.8) MS <sup>2</sup> [515]: 353 (100), 191 (13.8), MS <sup>3</sup> [515 → 353]: 191 (100) MS <sup>4</sup> [515 → 353 → 173]: 173 (39.2), 127 (100), 111 (56.1), 109 (22.0)	1,5- <i>O</i> -Dicafeoylquinic acid
17 <sup>a</sup>	12.9	243, 295, 325	515	MS <sup>2</sup> [515]: 354 (16.5), 353 (100) MS <sup>3</sup> [515 → 353]: 191 (100), 179 (48.7), 135 (10.5) MS <sup>4</sup> [515 → 353 → 191]: 173 (84.7), 127 (97.0), 111 (46.0), 93 (56.2), 85 (100)	3,5- <i>O</i> -Dicafeoylquinic acid
18	13.6	273, 332	461	MS <sup>2</sup> [461]: 446 (68.7), 341 (12.1), 299 (100), 284 (43.6) MS <sup>3</sup> [461 → 299]: 285 (20.5), 284 (100) MS <sup>4</sup> [461 → 299 → 284]: 284 (62.0), 283 (58.2), 255 (100)	Hispidulin-7- <i>O</i> -hexoside
19	14.2	300, 329	515	MS <sup>2</sup> [515]: 354 (11.3), 353 (100), 203 (10.8), 173 (17.5) MS <sup>3</sup> [515 → 353]: 191 (100), 179 (87.4), 173 (90.0) MS <sup>4</sup> [515 → 353 → 191]: 173 (59.1), 127 (100), 109 (22.6), 93 (30.0)	3- <i>O</i> -Caffeoylquinic acid hexoside
20	14.5	245, 300, 327	601	MS <sup>2</sup> [601]: 557 (69.7), 515 (82.5), 395 (100), 233 (33.4) MS <sup>3</sup> [601 → 395]: 335 (2.7), 233 (100), 173 (13.2) MS <sup>4</sup> [601 → 395 → 233]: 173 (100)	Malonyl-3,4- <i>O</i> -dicafeoylquinic acid
21	16.4	235, 300, 328	601	MS <sup>2</sup> [601]: 557 (57.3), 515 (10.6), 439 (15.5), 395 (100) MS <sup>3</sup> [601 → 395]: 335 (4.5), 234 (11.6), 233 (100), 173 (62.8) MS <sup>4</sup> [601 → 395 → 233]: 173 (100)	Malonyl-4,5- <i>O</i> -dicafeoylquinic acid
22	17.0	—	499	MS <sup>2</sup> [499]: 338 (11.8), 337 (100), 163 (17.4) MS <sup>3</sup> [499 → 337]: 191 (44.3), 173 (57.5), 163 (100) MS <sup>4</sup> [499 → 337 → 163]: 120 (14.4), 119 (100)	3- <i>O</i> - <i>p</i> -Coumaroyl-5- <i>O</i> -caffeoylquinic acid
23	17.8	—	425	MS <sup>2</sup> [425]: 179 (100), 135 (26.8) MS <sup>3</sup> [425 → 179]: 135 (100)	Caffeic acid diacylated hexoside
24	18.4	219, 315	499	MS <sup>2</sup> [499]: 354 (13.6), 353 (100), 337 (15.7), 191 (7.2) MS <sup>3</sup> [499 → 353]: 191 (100), 179 (36.5), 135 (19.1) MS <sup>4</sup> [499 → 353 → 191]: 173 (77.6), 155 (72.0), 127 (10.6), 95 (100)	3- <i>O</i> -Caffeoyl-5- <i>O</i> - <i>p</i> -coumaroylquinic acid
25		241, 300, 324	529	MS <sup>2</sup> [529]: 368 (16.6), 367 (100), 191 (11.3) MS <sup>3</sup> [529 → 367]: 191 (100), 173 (11.3), 134 (12.0) MS <sup>4</sup> [529 → 367 → 191]: 173 (87.9), 134 (100), 111 (28.9), 93 (24.5)	1- <i>O</i> -Caffeoyl-5- <i>O</i> -feruoylquinic acid
26	19.0	—	467	MS <sup>2</sup> [467]: 248 (16.1), 247 (100) MS <sup>3</sup> [467 → 247]: 200 (84.1), 199 (96.4), 161 (64.8), 121 (100)	Brevifolin derivative
27	19.7	—	499	MS <sup>2</sup> [499]: 338 (17.0), 337 (100) MS <sup>3</sup> [499 → 337]: 173 (100), 164 (16.4), 163 (46.7) MS <sup>4</sup> [499 → 337 → 173]: 93 (100)	3- <i>O</i> -Caffeoyl-4- <i>O</i> - <i>p</i> -coumaroylquinic acid
28	20.2	—	467	MS <sup>2</sup> [467]: 248 (15.4), 247 (100) MS <sup>3</sup> [467 → 247]: 199 (27.7), 161 (64.8), 135 (21.2), 121 (100)	Brevifolin derivative
29	20.6	—	529	MS <sup>2</sup> [529]: 368 (20.3), 367 (100), 349 (13.3), 191 (11.4) MS <sup>3</sup> [529 → 367]: 191 (28.4), 173 (100), 135 (16.3)	1 or 5- <i>O</i> -Caffeoyl-4- <i>O</i> -feruloylquinic acid
30	21.7	—	307	MS <sup>2</sup> [307]: 195 (100), 193 (44.6), 177 (35.4), 167 (14.7), 151 (14.3) MS <sup>3</sup> [307 → 195]: 152 (100)	Unknown
31	22.4	—	399	MS <sup>2</sup> [399]: 153 (100), 152 (40.2) MS <sup>3</sup> [399 → 153]: 109 (100)	Protocatechuic acid diacylated hexoside
32	23.5	—	541	MS <sup>2</sup> [541]: 523 (59.5), 497 (100), 481 (57.3), 479 (37.3)	Unknown

Table 3. (Continued)

No.	$t_R$ (min)	UV $\lambda_{max}$ (nm)	$[M-H]^-$ $m/z$	HPLC-DAD-ESI/MS <sup>n</sup> $m/z$ (% base peak)	Assigned identity
				MS <sup>3</sup> [541 → 497]: 479 (38.1), 327 (26.5), 279 (24.2), 225 (100)	
33	24.7	—	425	MS <sup>2</sup> [425]: 180 (10.4), 179 (100), 135 (13.9)	Caffeic acid diacylated hexoside
				MS <sup>3</sup> [425 → 179]: 136 (15.5), 135 (100)	
34	25.8	—	419	MS <sup>2</sup> [419]: 219 (15.4), 163 (13.8), 153 (100), 152 (27.8)	Procatechuic acid derivative
				MS <sup>3</sup> [419 → 153]: 109 (100)	
35	26.0	—	415	MS <sup>2</sup> [415]: 371 (54.2), 219 (15.7), 195 (46.1), 153 (34.7), 151 (100), 149 (20.9), 125 (17.2)	Unknown
				MS <sup>3</sup> [415 → 151]: 135 (100), 109 (57.8)	
36	27.2	—	409	MS <sup>2</sup> [409]: 163 (100)	<i>p</i> -Coumaric acid diacylated hexoside
				MS <sup>3</sup> [409 → 163]: 119 (100)	
37 <sup>a</sup>	28.7	240, 300, 328	677	MS <sup>2</sup> [677]: 516 (25.7), 515 (100)	3,4,5- <i>O</i> -Tricaffeoylquinic acid
				MS <sup>3</sup> [677 → 515]: 353 (100), 299 (24.9), 191 (13.4), 174 (19.3), 173 (26.5)	
				MS <sup>4</sup> [677 → 515 → 353]: 191 (34.7), 173 (100), 179 (50.3)	

<sup>a</sup> Comparison with a reference standard.

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

Figure 2. Chemical structures of phenolic compounds detected in *Helichrysum monizii*.

with UV data. Even so, five peaks remained unidentified; these are very minor components of the extract, as can be seen in the chromatogram shown in Fig. 1. Table 3 shows the analytical data: retention time ( $t_R$ ), wavelength of maximum absorbance ( $\lambda_{max}$ ), deprotonated molecular ions  $[M-H]^-$ , and major diagnostic fragment ions of the main peaks. Figure 2 represents the chemical structures of the compounds detected in *Helichrysum monizii*.

**Quinic acid derivatives.** Free quinic acid, compound **2** ( $t_R = 2.9$  min), was found as a relatively important component of the extract, as it happened for the previously analysed *Helichrysum* subspecies (Gouveia and Castilho, 2009, 2010). A total of 19 quinic acid derivatives were found in the present study, most of them as quinic acid structures esterified with acyl moieties. The determination of the exact location of acyl groups in the quinic acid structure was achieved based on the MS<sup>n</sup> data and taking into account the hierarchical key for the identification by LC-MS<sup>n</sup> of quinic acid derivatives reported before (Clifford *et al.*, 2005).

Mono-, di- and tricaffeoylquinic acids were identified by the use of standards: compounds **3** ( $t_R = 4.3$  min) as 3-*O*-caffeoylquinic acid, **5** ( $t_R = 5.0$  min) as 5-*O*-caffeoylquinic acid, **6** ( $t_R = 6.4$  min) as

1,3-*O*-dicaffeoylquinic acid, **15** ( $t_R = 12.0$  min) as 3,4-*O*-dicaffeoylquinic acid, **16** ( $t_R = 12.5$  min) as 1,5-*O*-dicaffeoylquinic acid, **17** ( $t_R = 12.9$  min) as 3,5-*O*-dicaffeoylquinic acid, and **37** ( $t_R = 28.7$  min) as 3,4,5-*O*-tricaffeoylquinic acid.

Compound **8** ( $t_R = 7.4$  min) exhibited a  $[M-H]^-$  ion at  $m/z$  367 and its MS<sup>2</sup> fragmentation gave, as base peak, a fragment ion at  $m/z$  191 [quinic acid- $H$ ]<sup>-</sup>. MS<sup>n</sup> data are consistent with those describe in literature for 5-*O*-feruloylquinic acid (Kuhnert *et al.*, 2010). Compound **10** ( $t_R = 7.9$  min) displayed a  $[M-H]^-$  ion at  $m/z$  675. The MS<sup>2</sup> fragmentation observed gave the main fragment ion at  $m/z$  337, demonstrating that the deprotonated molecular ion is a dimer of an ion with  $m/z$  of 337. Comparison of the MS<sup>n</sup> fragmentation data of this ion at  $m/z$  337 to those presented by Clifford *et al.* (2003) revealed the characteristic fragments of 5-*O*-*p*-coumaroylquinic acid: an MS<sup>3</sup> peak base at 181  $m/z$  and strong MS<sup>4</sup> ions at  $m/z$  85, 127 and 172. This substance was previously identified by our group in extracts of *Helichrysum melaleucum* (Gouveia and Castilho, 2010).

One more peak showed a  $[M-H]^-$  ion at  $m/z$  337 and occurred at a retention time of 7.0 min (compound **7**). This compound showed an MS<sup>n</sup> fragmentation behaviour and UV



spectra similar to those of 5-*O-p*-coumaroylquinic acid (**10**). Compound **7**, never found in the other *Helichrysum* subspecies, was tentatively identified as the *cis*-isomer, since often hydroxycinnamic acids occur with the cinnamic group in the *trans* configuration, attributed to **10**. The exposure at UV light may induce geometric isomerisation (Clifford *et al.*, 2006a). This isomerisation can occur due to exposure to UV light during sample workup or it can be from a biosynthetic origin. In order to confirm the identification of *cis*-isomer, *Helichrysum monizii* sample was irradiated with UV light for 3 h at room temperature and then analysed by LC-DAD-ESI/MS<sup>n</sup>. The peak area corresponding to compound **10** decreased substantially while the peak area of compound **7** increased, confirming **7** and **10** as a *cis/trans* pair of isomers of 5-*O-p*-coumaroylquinic acid.

Compound **19** occurred at a retention time of 14.2 min and showed UV absorption maxima at 300 and 329 nm, which are characteristic of caffeoylquinic acid conjugates. Despite that it gave a [M – H]<sup>–</sup> ion at *m/z* 515 and that an MS<sup>2</sup> fragment ion at *m/z* 353 was formed due to the loss of a neutral moiety of 162 Da, it was not identifiable as a dicaffeoylquinic acid isomer. The loss of 162 Da can suggest either a caffeoyl loss or a hexoside loss. The former possibility was discarded since in this work five of the six possible isomers of dicaffeoylquinic acid were positively identified by reference substances with the exception being 1,4-diCQA isomer. Auxiliary fragmentation of the ion at *m/z* 353 resulted in a fragment ion at *m/z* 191, as base peak, and a very intense fragment ion at *m/z* 179 (ca. 80% of base peak) signifying a 3-*O*-caffeoylquinic acid and rejecting the 1,4-diCQA hypothesis. Based on the MS<sup>n</sup> data, **19** was thus characterised as 3-*O*-caffeoylquinic acid hexoside. It was not possible to determine the exact hexoside linkage position, but it should not be directly linked to the caffeoyl moiety because no fragment ion at *m/z* 323 was detected.

Compounds **20** (*t<sub>R</sub>* = 14.5 min) and **21** (*t<sub>R</sub>* = 16.1 min) were identified as malonylcaffeoylquinic acids with an [M – H]<sup>–</sup> ion at *m/z* 601. Their MS<sup>2</sup> spectra showed main fragment ions at *m/z* 557 [M – H – 44]<sup>–</sup> and *m/z* 515 [M – H – 86]<sup>–</sup>, due to the malonyl moiety (Zhang *et al.*, 2007). The MS<sup>2</sup> base peak ion occurred at *m/z* 395 and corresponds to the loss of 206 Da (acetyl-caffeoyl). This type of fragmentation indicates that the malonyl group is linked to a caffeoyl moiety and not directly to the quinic acid structure. This type of compounds is not commonly found in plants but have been detected and described in our recent work with two endemic *Helichrysum* plants from Madeira (Gouveia and Castilho, 2009, 2010).

The dicaffeoylquinic acid part was characterised based on the fragmentation of MS<sup>2</sup> ion at *m/z* 515. Comparing the MS<sup>n</sup> fragmentation data, UV spectra and HPLC retention times these two compounds were identified as malonyl-3,4-*O*-dicaffeoylquinic acid (compound **20**) and malonyl-4,5-*O*-dicaffeoylquinic acid (compound **21**).

Compounds **22** (*t<sub>R</sub>* = 17.0 min), **24** (*t<sub>R</sub>* = 18.4 min) and **27** (*t<sub>R</sub>* = 19.7 min) all gave [M – H]<sup>–</sup> ions at *m/z* 499. For **22**, the MS<sup>2</sup> spectrum showed a fragment ion at *m/z* 337, suggesting the loss of a caffeoyl moiety (162 Da) in the first place. The MS<sup>3</sup> and MS<sup>4</sup> spectra base peaks corresponded to fragment ions at *m/z* 163 and 119, respectively. Thus, **22** was tentatively identified as 3-*O-p*-coumaroyl-5-*O*-caffeoylquinic acid. The first substituent group eliminated from **24** was a coumaroyl moiety (146 Da) forming a MS<sup>2</sup> spectrum base peak at *m/z* 353. The MS<sup>3</sup> fragmentation of this ion gave as main fragment a fragment ion at *m/z* 179 (ca. 36% of base peak) indicating a 3-*O*-caffeoylquinic acid structure. Therefore, **24** was characterised as 3-*O*-caffeoyl-

5-*O-p*-coumaroylquinic acid. Compound **27**, in a similar way of compound **22**, exhibited in the MS<sup>2</sup> spectrum, as base peak, a fragment ion at *m/z* 337 implicating a neutral loss of 162 Da (caffeoyl moiety). Subsequent MS<sup>n</sup> fragmentation of this ion gave a similar fragmentation pattern to that described for 4-*O-p*-coumaroylquinic acid (Clifford *et al.*, 2003). Therefore, **27** was identified as 3-*O*-caffeoyl-4-*O-p*-coumaroylquinic acid (Clifford *et al.*, 2006b).

At a retention time of 18.4 min, besides the presence of **24**, described above, there was a co-eluted compound, **25**. This was similar to compound **29** (*t<sub>R</sub>* = 20.6 min) in originating an [M – H]<sup>–</sup> ion at *m/z* 529. Literature reports (Han *et al.*, 2008) mention that this deprotonated molecular ion is indicative of a caffeoylferuloylquinic acid structure. The MS<sup>2</sup> spectrum base peak of both compounds **25** and **29** was a fragment ion at *m/z* 367 referent to the loss of 162 Da (caffeoyl moiety). The differences observed in the MS<sup>3</sup> spectra allowed the identification of these two isomers. The MS<sup>3</sup> base peak was a fragment ion at *m/z* 191 for **25** and *m/z* 173 for **29**, indicative of a 5-*O*-feruloylquinic acid and 4-*O*-feruloylquinic acid structure, respectively. The position of the caffeoyl moiety in **25** should be the 1-OH or 3-OH quinic acid's structure. Since it was not detected any fragment ion at *m/z* 179 with relative intensity above 50% of base peak, therefore the 3-OH position was not a probable linkage position. Consequently, **25** was identified as 1-*O*-caffeoyl-5-*O*-feruloylquinic acid.

Compound **29** has three positions available for the esterification of quinic acid with the caffeoyl group (1-OH, 3-OH and 5-OH). Based on the MS<sup>2</sup> fragmentation data, which revealed a fragment ion at *m/z* 191 but no fragment ion at *m/z* 179, the 1-OH and 5-OH positions are preferable. Also, it is more difficult for caffeoyl groups located in 3-OH positions to be the first moiety to be lost in the MS<sup>2</sup> fragmentation, as it was observed for **29**. Distinction between 1-OH or 5-OH position could only be achieved based on their differences of retention time or using a standard solution. Therefore, **29** was tentatively characterised as 1 or 5-*O*-caffeoyl-4-*O*-feruloylquinic acid (Gouveia and Castilho, 2009, 2010).

**Phenolic acids.** Among the several phenolic compounds detected in *Helichrysum monizii* four of them were characterised as caffeic acid derivatives. Compound **1** (*t<sub>R</sub>* = 2.9 min) displayed an [M – H]<sup>–</sup> ion at *m/z* 683 and in the MS<sup>1</sup> spectrum an intense fragment ion at *m/z* 341 was also observed. The MS<sup>2</sup> fragmentation of the ion at *m/z* 683 gave a fragment ion at *m/z* 341, indicating that the former ion is a dimer of the last one. The ion at *m/z* 341 under MS<sup>3</sup> fragmentation easily lost a neutral residue of 162 Da, originating a fragment ion at *m/z* 179. The ion at *m/z* 179 indicates a deprotonated caffeic acid ion confirmed by the MS<sup>4</sup> ions at *m/z* 161 [caffeic acid – H – H<sub>2</sub>O]<sup>–</sup> and *m/z* 135 [caffeic acid – H – CO<sub>2</sub>]<sup>–</sup>. The 162 Da neutral group expelled and described in the MS<sup>1</sup> fragmentation can be attributed to a caffeic acid or to a *O*-hexoside group. It is known that *O*-hexosides conjugates occur normally at lower retention times when compared to the sugar free compound. We analysed a standard solution of caffeic acid (*t<sub>R</sub>* = 7.0 min) and the retention time found was higher than that of compound **1**. Based on these results, we can infer this compound is more likely to be a caffeic acid-*O*-hexoside rather than a dicaffeic acid. The fragmentation scheme for caffeic acid-*O*-hexoside was recently proposed by Hossain *et al.* (2010).

Compound **9** (*t<sub>R</sub>* = 7.7 min) showed a [M – H]<sup>–</sup> ion at *m/z* 533 and its MS<sup>n</sup> fragmentation showed the characteristic fragment ions of caffeic acid such as *m/z* 179 and *m/z* 135 (caffeic acid – H – CO<sub>2</sub>). Therefore, **9** was characterised as a caffeic acid derivative.

Two more peaks were associated with caffeic acid derivatives, compound **23** ( $t_R = 17.8$  min) and compound **33** ( $t_R = 24.7$  min). They exhibited  $[M-H]^-$  ions at  $m/z$  425 and their  $MS^2$  fragmentation led to a fragment ion at  $m/z$  179 (loss of 246 Da). The remaining structure can be attributed to a diacetylated hexose (162 + 42 + 42 Da; Zhao *et al.*, 2008). Therefore, **23** and **33** were identified as caffeic acid diacetylated hexosides.

Compound **31** ( $t_R = 22.4$  min) gave a  $[M-H]^-$  ion at  $m/z$  399. Its  $MS^2$  fragmentation led to the loss of a neutral fragment of 246 Da (diacetylated hexoside) forming a fragment ion at  $m/z$  153. The

$MS^3$  fragmentation of this ion gave, as base peak, a fragment ion at  $m/z$  109 due to the loss of 44 Da, probably  $CO_2$ . These  $MS^n$  data point out to the hypothesis of a protocatechuic acid or a gentisic acid structure. The first hypothesis was supported by comparison with a standard solution of protocatechuic acid ( $MS^n$  data not shown) where it was possible to detect the same fragments. Hence, **31** was identified as protocatechuic acid diacetylated hexoside.

Compound **34** ( $t_R = 25.8$  min) exhibited an  $[M-H]^-$  ion at  $m/z$  419 and its  $MS^2$  spectrum showed, as base peak, a fragment ion at  $m/z$  153, resulting from the loss of 266 Da. the ion at  $m/z$  153

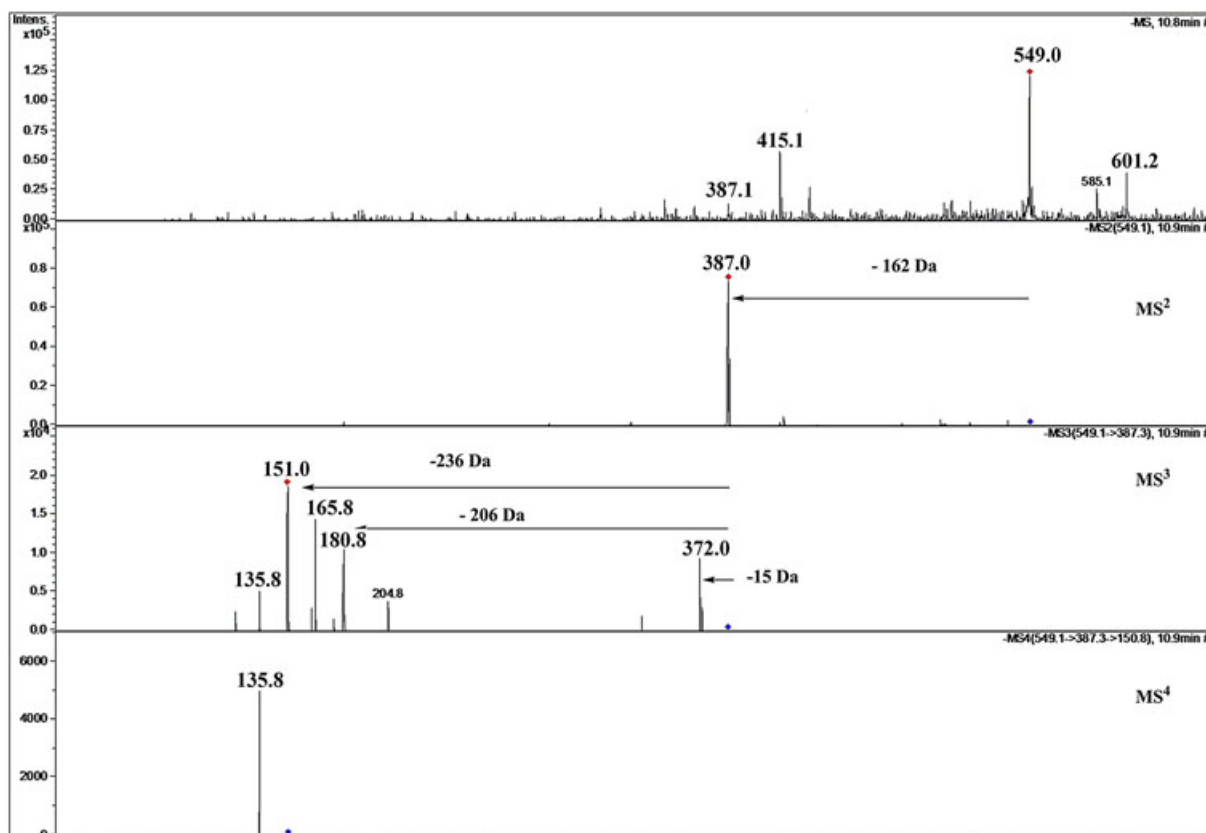


Figure 3. ESI/ $MS^n$  negative mode analysis of compound **14**. Sequential fragmentation,  $MS^n$  ( $n = 1-4$ ) of the ion at  $m/z$  549.

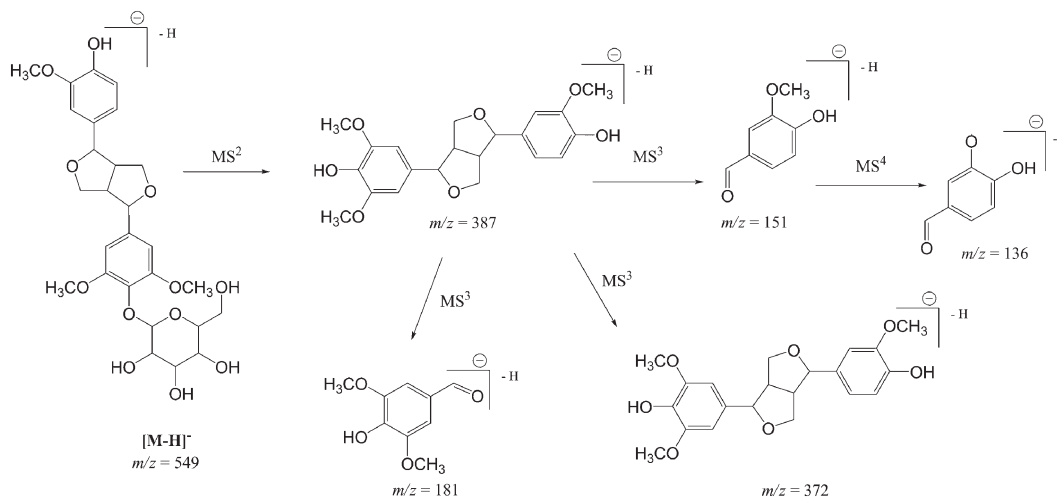


Figure 4. Proposed fragmentation pathway for compound **14**.

was characterised as [protocatechuic acid–H]<sup>–</sup> for the same reasons as mentioned for **31**. However, the remaining structure could not be established and **34** was identified as a protocatechuic acid derivative.

### Other compounds

Compound **13** ( $t_R$  = 10.0 min) showed a [M–H]<sup>–</sup> ion at  $m/z$  477 and was identified as isorhamnetin-*O*-hexoside. This compound is a flavone and in the MS<sup>2</sup> fragmentation the hexoside residue was lost to give the aglycone ion (Y<sub>0</sub><sup>–</sup>) at  $m/z$  315. Subsequent MS<sup>*n*</sup> fragmentation was very similar to that described in literature for isorhamnetin (Gouveia and Castilho, 2010).

Compound **14** ( $t_R$  = 10.8 min) displayed an [M–H]<sup>–</sup> ion at  $m/z$  549 and under MS<sup>2</sup> fragmentation lost a 162 Da neutral fragment originating a fragment ion at  $m/z$  387. Further MS<sup>*n*</sup> fragmentation gave the MS<sup>3</sup> and MS<sup>4</sup> base peaks at  $m/z$  151 and  $m/z$  136, respectively. Comparing these results to those described by (Eklund *et al.*, 2008), **14** was identified as a furofuranolignan hexoside.

In this type of compounds the occurrence of the fragment ions at  $m/z$  151 (guaiacyl) and/or 181 (syringyl) are due to the cleavage between the  $\alpha$ - and  $\beta$ -position in the side chain. However, an [M–H–162–15]<sup>–</sup> at  $m/z$  372 was also observed with high intensity (ca. of base peak) that corresponds to the loss of a methoxyl group (Fig. 3). The presence of a hexoside moiety is attributed to the low retention time of this compound but its precise location could not be determined. Therefore, **14** was tentatively identified as medioresinol-*O*-hexoside (Fig. 4).

Compound **18** ( $t_R$  = 13.6 min) exhibited an [M–H]<sup>–</sup> ion at  $m/z$  461 and UV maximum absorption bands at 272 and 332 nm. The MS<sup>2</sup> fragmentation showed the loss of a hexoside residue, originating a fragment ion at  $m/z$  299. This behaviour was found in our recent studies with *Helichrysum* species and **18** was identified hispidulin-7-*O*-hexoside.

Compounds **26** ( $t_R$  = 19.0 min) and **28** ( $t_R$  = 20.2 min) possessed the same [M–H]<sup>–</sup> ion at  $m/z$  467. The MS<sup>2</sup> spectra showed a fragment ion at  $m/z$  247, as base peak. The nature of this moiety of 220 Da could not be identified based only on these MS<sup>*n*</sup> data. For instance, the ion at  $m/z$  247 was identified as brevifolin, a polyphenol (He and Xia, 2007). Therefore, **26** and **28** were identified as brevifolin derivatives.

Compound **36** ( $t_R$  = 27.2 min) gave a [M–H]<sup>–</sup> ion at  $m/z$  409 and under MS<sup>*n*</sup> fragmentation lost a neutral fragment of 246 Da, resulting in a fragment ion at  $m/z$  163. MS<sup>3</sup> fragmentation revealed characteristic ions of *p*-coumaric acid, for example MS<sup>3</sup> ion at  $m/z$  119 (loss of CO<sub>2</sub>) (comparison made with a standard solution). As described above, the 246 Da fragment can be associated with a diacylated hexoside moiety. Thus, **36** was tentatively identified as *p*-coumaric acid diacylated hexoside.

Overall, dicaffeoylquinic acids are, by far, the most abundant components of the methanol extracts of *Helichrysum monizii* and should be responsible for its exceptionally high antioxidant properties.

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