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Validation of a HPLC-DAD–ESI/MS^n method for caffeoylquinic acids separation, quantification and identification in medicinal *Helichrysum* species from Macaronesia

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ABSTRACT

The caffeoylquinic acids (CQA) found in *Helichrysum* species from Madeira Archipelago were identified and quantified. The HPLC-DAD–ESI/MS^n method applied was developed and validated showing a good repeatability with recovery values ≥96%. The use of a RP-C_{18} with a small internal diameter (Ø 3.0 mm) and an acidic mobile phase (acetonitrile and water with 0.1% (v/v) of formic acid) enabled the separation of all compounds within a 30 min analysis. A good resolution between 1,5-*O*-diCQA and 3,5-*O*-diCQA isomers, usually hard to separate, was also accomplished.

Dicaffeoylquinic acids isomers were the major components among the quantified hydroxycinnamic acids. 1,5-*O*-diCQA, 3,5-*O*-diCQA and 5-*O*-CQA were the compounds found in higher amounts for the different species. The distinct uses of these plants described in the local folk medicine can be related to the phenolic composition.

1. Introduction

Chlorogenic acids (CGAs) are a family of esters formed between *trans*-cinnamic acids, such as caffeic, ferulic and *p*-coumaric acids, and (−)-quinic acid derived from the shikimic acid pathway (Clifford, Johnston, Knight, & Kuhnert, 2003). This is a ubiquitous group of compounds, for which green coffee is the main source, with a content of 5–12 g 100 g^{-1} (Farah, Monteiro, Donangelo, & Lafay, 2008). Besides coffee, other plants, in particular of the Asteraceae family, have been reported as important sources of CGAs (Jaiswal, Deshpande, & Kuhnert, 2011; Jaiswal, Kiprotich, & Kuhnert, 2011; Jaiswal, Sovdat, Vivan, & Kuhnert, 2010).

Caffeoylquinic acids (CQA) are generally involved in plant responses to biotic and abiotic stresses and 5-*O*-caffeoylquinic acid (5-*O*-CQA), is an intermediate in the lignin biosynthesis pathway (Mondolot et al., 2006).

There are several advantageous health properties associated to this class of compounds, such as antioxidant capacity, antiviral, antibacterial, anti-inflammatory, reduction of the relative risk of cardiovascular disease, diabetes type 2 and Alzheimer's disease (Farah et al., 2008).

Di- and tri-esters of quinic acids containing gallic or caffeic acid showed relevant anti-HIV properties. DiCQA isomers, in particular, are currently under close scrutiny since they have been found to be a novel class of antiviral substances, namely as integrase inhibitors, being potent inhibitors of HIV-1 replication, both in cultured cell lines and in catalytic activity of integrase in vivo (Hu, Chen, Dong, & Southerland, 2010).

3,5-*O*-diCQA was also found to be a potent inhibitor of respiratory syncytial virus (RSV) *in vitro* and *in vivo* (Ojwang et al., 2005) and to have neuroprotective effects on hydrogen peroxide-induced cell death in SH-SY5Y cells (Kim, Park, Jeon, Kwon, & Chun, 2005). 1,3-*O*-diCQA (cynarin) and its precursor isomer 1,5-*O*-diCQA are consider the responsible compounds for the hypocholesterolemic activity attributed to artichoke (Bundy, Walker, Middleton, Wallis, & Simpson, 2008; Coon & Ernst, 2003). Also, 1,5-*O*-diCQA is known for being a potent and non toxic HIV-1 integrase inhibitor and its anti-HIV activity *in vitro* and *in vivo* against a variety of animal experimental models has been established (Gu, Dou, Wang, Dong, & Meng, 2007).

DiCQA isomers are also known to possess higher antioxidative, tyrosinase inhibitory and antiproliferation activities when compared to monoCQA (Iwai, Kishimoto, Kakino, Mochida, & Fujita, 2004).

Despite that different activities are associated with different isomers, it is usual to quantify CQA compounds as a group and not as individual components. Miketova, Schram, Whitney, Kearns, and Timmermann (1999) reported structure-activity relationships for CQA isomers towards HIV. Thus, it seems important not only to detect and identify the different CQA isomers present in a plant extract matrix but also to quantify them, a task not always easy even with access to modern techniques, due to problems of great similarity of spectra and to the common co-elution of these compounds.

In our recent work, the phenolic composition of endemic species of the *genus Helichrysum* (*Asteraceae*) existing in Madeira Archipelag (Macaronesia), has been studied (Gouveia & Castilho, 2009, 2010, 2011,

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2011b). Among the wide variety of compounds detected and characterized there were flavonoids and phenolic acids. Several isomers of mono-, di- and triCQA isomers have been found as major components.

The *genus Helichrysum* comprises more than 500 species distributed through the world. Several biological activities, such as antimicrobial, anti-allergic, antioxidant, anti-inflammatory, cough relief, cold and wounds have been associated to plants belonging to this genus (Al-Rehaily, Albishi, El-Olemy, & Mossa, 2008; Carini, Aldini, Furlanetto, Stefani, & Facino, 2001).

The endemic species in Madeira Archipelago are *Helichrysum devium* Johns., *Helichrysum melaleucum* Rchb. Ex Holl; *Helichrysum obconicum* DC. E and *Helichrysum monizii* Lowe (Jardim & Francisco, 2000). The first three of these subspecies are used traditionally for medicinal proposes but recently chefs from high class hotels have shown interest in using these new flavors as a neutraceuticals in herbal teas and salads. Infusions of the total aerial parts of *H. devium* and *H. melaleucum* are used for the treatment of bronchitis and pharyngitis while infusions of the flowers from *H. melaleucum* are used as cardiotonic and cough relief remedy. *H. obconicum* aerial parts are used as herbal teas as digestive, stomachic and for intestinal diseases (Rivera & Obón, 1995). *H. monizii* is an extremely rare plant which inhabits only a few cliffs on south coast of Madeira and therefore no ethnopharmacological information is available to this plant.

Since these plants are used for different health ailments by local traditional medicine, it was considered interesting to determine the accurate (quantitative) composition of their phenolic profile, composed mainly of caffeoylquinic acids isomers. Therefore, the aim of this work was to separate, indentify and quantify the mono-, di- and triCQA compounds, present in *Helichrysum* species from Madeira.

Several papers described the quantification and identification of caffeoylquinic acid isomers in crude plant extracts such as sunflower (Papetti et al., 2008; Weisz, Kammerer, & Carle, 2009) and fennel (Krizman, Baricevic, & Prosek, 2007; Parejo, Viladomat, Bastida, & Codina, 2004). However, the retention times of the compounds are substantially higher than those presented in our study. Furthermore none of those studies reported the simultaneous separation of the isomers herein described.

The novelty of this work emerges from the development and validation of a HPLC-DAD–ESI/MS^n method using a narrow reversed phase C_{18} column to separate the CQA compounds including the 1,5-*O*-diCQA, 3,4-*O*-diCQA and 3,5-*O*-diCQA isomers that are very difficult to separate with less than 30 min of analysis. Peak identity and purity were checked and confirmed by tandem mass spectrometry.

2. Material and methods

2.1. Reagents and materials

5-*O*-Caffeoylquinic acid (99%) was purchased from Acros Organics (Geel, Belgium) and 1,3-*O*-dicaffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, 4,5-*O*-dicaffeoylquinic acid and 3,4,5-*O*-tricaffeoylquinic acid were obtained from Chengdo Biopurity Phytochemicals, Ltd China (Sichuan, China).

HPLC–MS^n grade acetonitrile (99.9%) was obtained from Labscan (Gliwice, Poland), ultra-pure water (Milli-Q Waters purification system, USA) and formic acid (analytical grade) were used for mobile phase preparation in the HPLC-DAD–ESI/MS^n analysis. The methanol used for extraction of *Helichrysum* was AR grade, purchased from Labscan (Gliwice, Poland). Eluents prepared for HPLC-DAD–ESI/MS^n analysis were additionally filtered through 0.45 μm Nylon micropore membranes.

2.2. Standard solutions

Stock standard solutions (1000 μg mL^{-1}) of each analyte were prepared in methanol and stored in a refrigerator at −20 °C until use.

2.3. Plant material and sample preparation

The plant material analyzed in the present study consisted of four *Helichrysum* endemic species from Madeira Archipelago.

Samples of *H. devium*, *H. melaleucum*, *H. obconicum* were collected by the authors in the wild and identified by taxonomist Fátima Rocha, from the Madeira Regional Secretary for the Environment and Agriculture; a voucher was deposited in the Madeira Botanical Garden Herbarium collection. Sample of *H. monizii* was obtained from Madeira Botanical Garden endemic plant collection.

When enough plant material was available, assessment of morphological parts was performed for the leaves, flowers and stems that were collected and stored separately.

Dried and powdered plant material (100 g) was extracted by maceration (solid–liquid extraction) 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 10 mg mL^{-1} were prepared by dissolving dried extract in initial HPLC mobile phase without formic acid (acetonitrile: water, 20:80 (v/v)).

These solutions were filtered through 0.45 mm Nylon micropore membranes prior to use and 10 μL were injected for HPLC-DAD/ESI–MS^n analysis.

2.4. HPLC-DAD–ESI/MS^n analysis

2.4.1. Instrumentation

Analysis were performed on a Dionex ultimate 3000 series instrument (California, USA) coupled to a binary pump, a diode-array detector (DAD), an automatic injector, an autosampler and a column compartment (at 20 °C). A Phenomenex Gemini C_{18} column (5 μm, 250 × 3.0 mm i.d., Phenomenex) was used for all separations.

The mobile phase consisted of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). The gradient program was used as follows with a total analysis time of 40 min: 80% A (0–1 min), 78% A (8–10 min), 76% A (12–14 min), 75% A (16–18 min), 73% A (20 min), 50% A (30 min), 0% A (31–35 min), and 80% B (36–40 min). The flow rate was 0.4 mL min^{-1} and the injection volume was 10 μL. UV-DAD detection was performed at λ = 320 nm.

For HPLC–ESI/MS^n analysis, a Bruker Esquire model 6000 ion trap mass spectrometer (Bremen, Germany) fitted with an ESI source was used operating in the negative mode. Esquire control software was used for the data acquisition and Data Analysis for processing. Negative ion mass spectra of the column eluate were recorded in the range *m/z* 100–1000 at a scan speed of 13,000 Da s^{-1}. High purity nitrogen (N_2) was used both as drying gas at a flow of 10.0 mL min^{-1} and as a nebulizing gas at a pressure of 50 psi. The nebulizer temperature was set at 365 °C and a potential of +4500 eV was used on the capillary. Ultra-high-purity helium (99.99%) (He) was used as collision gas at a pressure of 1×10^{-5} mbar and the collision energy was set at 40 eV.

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

The method described above was proposed for the first time and, after its validation, was applied to our samples of interest.

2.4.2. Analytical curve

An external standard method was used for quantification of CQA compounds. Working standard solutions were prepared daily by dilution of the stock solutions with the initial eluent gradient in the concentration range 0.1–700 μg mL^{-1}.

The analytical curve was determined on six levels of concentration with three injections per level. HPLC chromatogram peak areas were plotted against the known concentrations of the standard solutions to

establish calibration equations. A linear regression equation was calculated by the least squares method.

2.5. Validation parameters

2.5.1. Selectivity and peak purity

Selectivity was checked by using an extract of each sample to be analyzed and a mixture of available standards optimizing separation and detection.

The purity of the peaks was verified by DAD ($\lambda = 200–600$ nm) by the Chromeleon PPI match factor. The ideal match factor is 1000.

2.5.2. Linearity, limits of detection and quantification

The linearity was checked in three replicates in this range at least in six points. HPLC-DAD (320 nm) chromatogram peak areas were plotted against the known concentrations of the standard solutions to establish calibration equations. A linear regression equation was calculated by the least squares method.

Limits of detection and quantification were determined based on the standard deviation of the response and the slope. The limit of detection (LOD) was expressed as: $LOD = \frac{3.3\sigma}{S}$ and the limit of quantification (LOQ) as: $LOD = \frac{10\sigma}{S}$ where σ is the standard deviation of the response and S the slope of the analytical curve (Communities, 1996).

2.5.3. Precision

Three different concentrations of each standard (700, 300 and 2.5 μg mL^{-1}) were used for intra- and interday precision.

The repeatability precision data were calculated by the areas of 5 replicates of each concentration on the same day (%RSD intraday). The areas of the three consecutive injections performed at each concentration on 3 different days were used to calculate %RSD interday repeatability (%RSD interday).

2.5.4. Accuracy

The accuracy of the method was evaluated by analyzing the percentage of recovery of each standard. A sample of *H. devium* flowers was spiked with solutions (30 μg mL^{-1}) of each standard compound. The spiked solutions were analyzed in triplicate under the same conditions described above.

Recovery was expressed as the percent mean ratio of the measured added concentration to nominal value.

3. Results and discussion

3.1. Chromatographic separation optimization

Preliminary tests were performed to obtain the best peak resolution and separation of the six compounds, for which both a mixture of standards and a real sample (*H. devium* flowers extract) were used.

The addition of 0.1% (v/v) of formic acid to mobile phase resulted in a better separation of three isomers initially overlapped (3,4-, 1,5- and 3,5-*O*-dicaffeoylquinic acid) and also after the column separation and UV detection the compounds could be analyzed by HPLC–ESI/MS^n tandem mass spectrometry.

A Phenomenex Gemini C_{18} column (5 μm, 250 × 4.6 mm i.d., Phenomenex) was first employed and the flow rate used was 1 mL min^{-1}. A second column with the same packing material but with a smaller internal diameter was tested with the same samples and using a flow rate of 0.4 mL min^{-1}. Indeed, the same resolution could be achieved with the second column (Phenomenex Gemini C_{18} column: 5 μm, 250 × 3.0 mm i.d., Phenomenex) and the retention time for each compound was lower and solvent consumption was 60% lower. For this reason, this second column was selected for the validation of the method.

The best separation of all compounds is presented in chromatogram at Fig. 1 for a mixture of standards and a *Helichrysum* sample (*H. devium*).

3.2. Method validation

The HPLC method used was validated by evaluating the peak purity, linearity, limits of quantification and detection, precision and accuracy.

The precision in the retention time was also measured for qualitative intentions. A high repeatability in the retention time was obtained with (%RSD) values lower than 1% for both standards and extracts (data not shown).

The peak purity was determined for all the peaks and impurities and co-elutions were not observed (match factor of 1000 in all samples).

Linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy were evaluated for quantification purposes (Table 1).

LOD values ranged from 0.76 to 5.95 μg mL^{-1} and the LOQ values from 2.29 to 18.02 μg mL^{-1} suggesting good capacity of this method for the quantification of each of the compounds under study.

Correlation coefficients (R^2) were higher than 0.999 over the concentration range confirming the linearity of the method for each compound. The relative standard deviation (%RSD) showed good repeatability, with values between 0.09% and 2.84%.

The precision of the method for each standard was determined for three concentrations covering the quantification range values. Five non-consecutive injections for each concentration were performed on the same day. The %RSD for the intraday repeatability ($n = 15$) are given in Table 1.

The %RSD of interday precision was evaluated, at the same concentrations used for intraday precision, based on three consecutive injections on three different days ($n = 27$) (Table 1).

Accuracy measures the effectiveness of all the analytical steps performed including the extraction process; therefore all extractions should be performed as closely as possible (Parejo et al., 2004). Since there is no reference material, the determination of accuracy becomes quite difficult.

The accuracy was measured from recovery studies of each standard in a spiked sample of *H. devium* flowers. The high recovery values (≥96%) associated with lower %RSD indicated a very good accuracy for this method.

3.3. Identification and quantification

The identification of CQA isomers in *Helichrysum* endemic species from Madeira was previously performed and published (Gouveia & Castilho, 2009, 2010).

In the present work, the chromatographic conditions were optimized as described above in order to obtain a good separation of the CQA isomers found in *Helichrysum* species.

The UV spectra of compounds containing a hydroxycinnamic moiety are characterized by maximum absorptions bands at 320–329 nm and 242 nm and a shoulder at 295–300 nm (Mabry, Markham, & Thomas, 1970).

The seven standard compounds were easily detectable at a wavelength of 320 nm (Fig. 1B).

The HPLC-DAD profiles of *Helichrysum* samples were also recorded at a wavelength of 320 nm and as example, Fig. 1A shows the chromatogram, at 320 nm, of *Helichrysum devium* roots extract.

CQA isomers were identified in each sample by comparison of both retention times and UV spectra with those of available standard compounds and were further confirmed by HPLC–ESI/MS^n analysis (Table 2); their chemical structures are given in Table 3.

The linkage position of caffeoyl groups to the quinic acid can be determined by the analysis of the MS^n fragmentation of the deprotonated molecular ion, $[M–H]^-$ and following the hierarchical key, that allows for the identification of a large number of CQA conjugates by their HPLC–ESI/MS^n fragmentation behavior, proposed by Clifford and co-workers work (Clifford, Knight, & Kuhnert, 2005; Clifford,

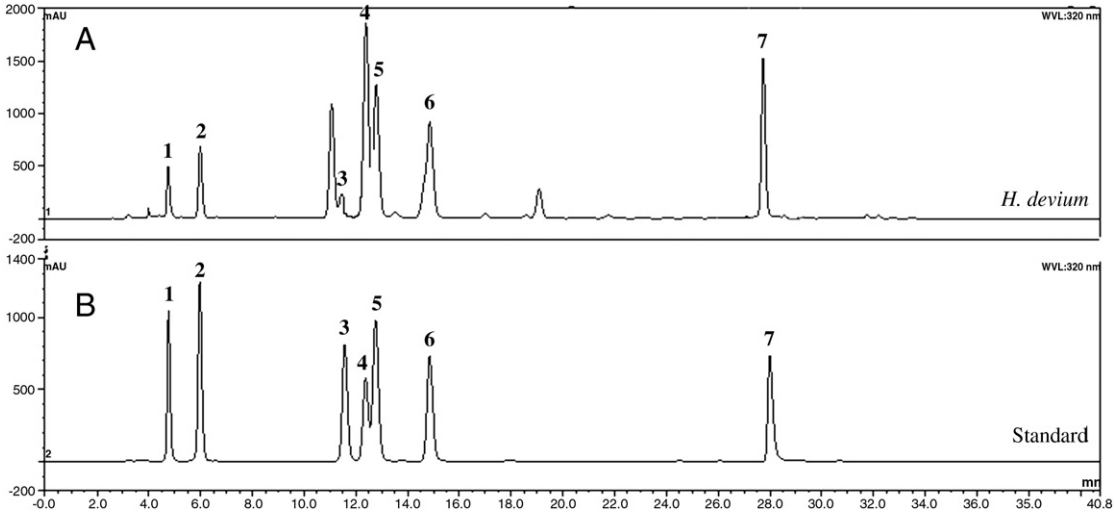


Fig. 1. HPLC-DAD chromatograms (λ = 320 nm) from: A) *Helichrysum devium* roots sample and B) a standard solution. Peak identification: (1) 5-*O*-caffeoylquinic acid; (2) 1,3-*O*-dicaffeoylquinic acid; (3) 3,4-*O*-dicaffeoylquinic acid; (4) 1,5-*O*-dicaffeoylquinic acid; (5) 3,5-*O*-dicaffeoylquinic acid; (6) 4,5-*O*-dicaffeoylquinic acid; and (7) 3,4,5-*O*-tricaffeoylquinic acid.

Marks, Knight, & Kuhnert, 2006; Clifford et al., 2003). In general, for monoCQA, the [quinic acid–H]$^-$ ion at *m/z* 191 appears as MS^2 spectrum base peak when the acyl group is linked to the 3-OH or 5-OH position; these two isomers can be further differentiated since the MS^2 [caffeic acid–H]$^-$ ion at *m/z* 179 is more significant for 3-OH compounds.

When the acyl group is connected to 4-OH, the MS^2 [quinic acid–H_2O–H]$^-$ ion at *m/z* 173 will appear as the base peak. These evidences can be observed in the MS^n spectra of 3,4-*O*-diCQA (compound **3**, Fig. 2) after the MS^1 lost of a caffeoyl unit connected to the 3-OH position.

The results of the quantitative analysis of *Helichrysum* species for their hydroxycinnamic acid contents are given in Table 4 and the values are expressed as mg 100 g^{-1} of dried plant.

The total amount of CQA in each sample was calculated by summarizing individual amounts of all constituents and ranged from 35.81 ± 0.12 mg 100 g^{-1} (*H. devium* stems) to 1367.9 ± 4.6 mg 100 g^{-1} (*H. melaleucum* leaves).

1,5-diCQA was found to be the CQA compound present in highest amounts in *H. devium*, *monizii* and *melaleucum* (with exception of the stems from *H. melaleucum*).

For *H. obconicum* samples the 3,5-diCQA isomer was found to be the CQA isomer present in highest amounts.

The values obtained for 1,5-diCQA content varied between 9.56 ± 0.039 mg 100 g^{-1} (*H. obconicum* total aerial parts) to 915.4 ± 1.4 mg 100 g^{-1} (*H. melaleucum* leaves)

The extracts of *H. melaleucum* are the ones with higher amounts of 1,5-diCQA in particular in the leaves with a value of 915.4 ± 1.4 mg

5-CQA is the second most abundant compound in all samples of *Helichrysum* analyzed and the *H. monizii* extract gave the highest content at 375.9 ± 0.53 mg 100 g^{-1} (*ca.* 32% of the total amount of CQA compounds). In fact for *H. melaleucum* stems, this was the main compound representing about 47% of the total amount of CQA.

The levels and relative amounts found for each of these compounds are comparable to those reported for other plants with high amounts of CQA isomers such as fennel (Parejo et al., 2004), sunflower (Weisz et al., 2009) and wormwood (Gouveia & Castilho, 2011b).

There are not many studies concerning to the quantification of these compounds in *Helichrysum* species. For example, for *H. italicum*, 5-*O*-CQA and 1,5-*O*-diCQA were found to be the main phenolic compounds representing 60% of the total phenolic composition (Zapesochnaya et al., 1992).

5-*O*-CQA, 1,5-*O*-diCQA and 4,5-*O*-diCQA were identified in all studied samples.

4,5-*O*-diCQA was present in amounts significantly lower comparing to the others compounds (10 fold less) and the sample with highest quantity of this compound was the roots of *H. devium* with a value of 68.78 ± 0.069 mg 100 g^{-1}.

As can be seen in Table 4, 3,5-*O*-diCQA is the main compound found in *H. obconicum* samples with values ranging from 56.53 ± 0.49 mg 100 g^{-1} (total aerial parts, *ca.* 33% of the total amount of CQA) to 469.5 ± 0.25 mg 100 g^{-1} (leaves, *ca.* 77% of the total amount of CQA). However, *H. monizii* was the species with higher content of 3,5-diCQA with a value of 303.3 ± 0.61 mg 100 g^{-1} (*ca.* 26% of the total amount of CQA).

Table 1
Regression curves, linearity, limit of detection (LOD), limit of quantification (LOQ), recovery and precision by HPLC-DAD analysis.

Compound	t_R (min)	Regression equation Y = mX + b		$R^2 \pm SD$	Concentration range (μg mL^{-1})	LOD μg mL^{-1}	LOQ μg mL^{-1}	Recovery (%)[b]	Repeatability (% RSD) (n = 15)			Interday precision (%RSD) (n = 27)		
		$m \pm SD$[a]	$b \pm SD$[a]						A	B	C	A	B	C
5-CQA	4.80	0.5371 ± 2.08^{-4}	1.878 ± 0.0615	0.9992 ± 5.80^{-5}	3–870	5.95	18.02	98.34 ± 1.45	0.79	1.67	0.50	0.65	1.46	0.56
1,3-diCQA	6.08	1.439 ± 9.50^{-4}	0.7958 ± 0.0697	0.9998 ± 1.53^{-4}	0.1–700	1.25	2.80	100.29 ± 1.82	0.54	1.18	2.08	1.34	1.44	1.52
3,4-diCQA	11.96	1.167 ± 1.56^{-2}	1.846 ± 0.151	0.9997±	0.1–700	1.39	4.21	97.34 ± 0.94	1.88	1.36	1.50	1.38	1.21	1.12
1,5-diCQA	12.70	0.9046 ± 9.32^{-3}	0.4744 ± 0.0120	0.9996 ± 1.01^{-4}	0.1–700	1.12	3.38	98.71 ± 1.05	1.19	0.69	2.78	0.93	0.67	1.83
3,5-diCQA	13.13	1.352 ± 2.23^{-3}	4.994 ± 0.212	0.9991 ± 1.15^{-4}	2.5–700	0.76	2.29	96.38 ± 0.89	0.25	0.58	1.88	0.57	0.53	1.64
4,5-diCQA	14.89	1.208 ± 7.95^{-3}	0.004700 ± 1.3^{-4}	0.9994 ± 5.77^{-5}	1–700	1.50	4.84	98.30 ± 1.02	0.62	0.12	2.10	0.46	0.38	1.53
3,4,5-triCQA	27.90	1.3041 ± 3.18^{-3}	7.866 ± 0.349	0.9991 ± 5.64^{-5}	1–700	2.48	4.50	96.24 ± 1.93	0.09	0.40	1.79	0.13	0.45	1.30

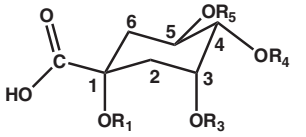
[a] Values are the mean of 3 replicates ± S.D.
[b] Only determined for *Helichrysum devium* flowers; concentrations: A—297.62; B—74.41; C—2.5 μg mL^{-1}.

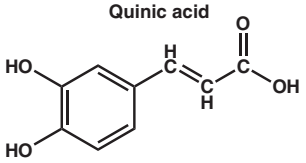
Table 2
Retention times, UV spectra and main characteristic ions obtained by HPLC-DAD–ESI/MS^n of caffeoylquinic acids isomers.

No.	t_R (min)	UV λ_{max} (nm)	$[M-H]^-$ m/z	Diagnostic ions (m/z)	Identification
1	4.81	242, 298 sh, 326	353	191, 173, 127, 109	5-*O*-Caffeoylquinic acid
2	6.08	243, 300 sh, 321	515	353, 191, 179, 135, 109	1,3-*O*-Dicaffeoylquinic acid
3	11.96	246, 300 sh, 323	515	353, 191, 173, 135, 111	3,4-*O*-Dicaffeoylquinic acid
4	12.84	243, 300 sh, 328	515	353, 191, 173, 127	1,5-*O*-Dicaffeoylquinic acid
5	13.13	243, 300 sh, 327	515	353, 191, 179, 127	3,5-*O*-Dicaffeoylquinic acid
6	15.03	243, 300 sh, 328	515	353, 173, 127, 93	4,5-*O*-Dicaffeoylquinic acid
7	28.07	240, 300sh, 329	677	515, 353, 173	3,4,5-*O*-Tricaffeoylquinic acid

Table 3
Chemical structures of the caffeoylquinic acids quantified.

No.	Compound
1	5-*O*-Caffeoylquinic acid
2	1,3-*O*-Dicaffeoylquinic acid
3	3,4-*O*-Dicaffeoylquinic acid
4	1,5-*O*-Dicaffeoylquinic acid
5	3,5-*O*-Dicaffeoylquinic acid
6	4,5-*O*-Dicaffeoylquinic acid
7	3,4,5-*O*-Tricaffeoylquinic acid





For some samples, a few compounds were only detected in trace level (below the LOQ) or were not detected at all.

The amounts of 3,4,5-*O*-triCQA provided by the analyzed *Helichrysum* species ranged between 4.630 ± 0.0032 and 10.58 ± 0.0076 mg 100 g^{-1} with exception of *H. devium* roots that revealed a 10 times higher value, at 57.59 ± 0.22 mg 100 g^{-1}.

H. monizii and *H. obconicum* samples revealed the highest quantity of 3,4-*O*-diCQA with values between 16.52 ± 0.045 and 18.96 ± 0.25 mg 100 g^{-1}. However, only in the *H. obconicum* total aerial parts extract, this compound was found in appreciably amounts with *ca.* 11% of its composition. For the other samples the percentage of 3,4-*O*-diCQA varied between 0.45% and 3.3%.

1,3-*O*-diCQA (usually known as cynarin) is a minor compound in the *Helichrysum* species, with highest amount in the roots of *H. devium* (18.02 ± 0.052 mg 100 g^{-1}). Among all the analyzed total aerial parts extracts only *H. monizii's* showed trace levels of this compound.

A recent study (Danino, Gottlieb, Grossman, & Bergman, 2009) revealed that 1,3-*O*-diCQA has a lower antioxidant IC_{50} value when compared to other standard antioxidant compounds (Trolox, ascorbic acid, caffeic acid and ferulic acid). Therefore, even present in very low amounts in the studied samples, this compound can greatly contribute to the strong total antioxidant activity of these plants (research under progress, data to be published elsewhere).

The HPLC–ESI/MS^n screening did not revealed other peaks with typical MS^n fragment ions of CQA isomers. Therefore, it is possible to infer that the majority of the hydroxycinnamic compounds of *Helichrysum* species are diCQAs isomers, and these ethnobotanical species may be promising sources of these specific isomers.

The contribution of individual compounds to the global biological activities of plant extracts can be substantially different. This could explain the distinct uses of these *Helichrysum* species in the traditional medicine of Madeira.

H. devium and *H. melaleucum* are both used as herbal teas or alcoholic infusions in the treatment of respiratory diseases, such as bronchitis and pharyngitis. The high levels of 1,5-*O*-diCQA observed for these two species can be related to these properties. *H. obconicum*, locally used as an herbal tea for digestive, stomachic and intestinal problems, presented 3,5-*O*-diCQA as main compound in contrast to the two other species mentioned above.

In a recent study of our group (Gouveia & Castilho, 2011a), we analyzed *Artemisia argentea*, a plant used for stomachic problems and we found out that similar to *H. obconicum* 3,5-*O*-diCQA was the most abundant CQA isomer (but lower levels than those found for *H. obconicum*).

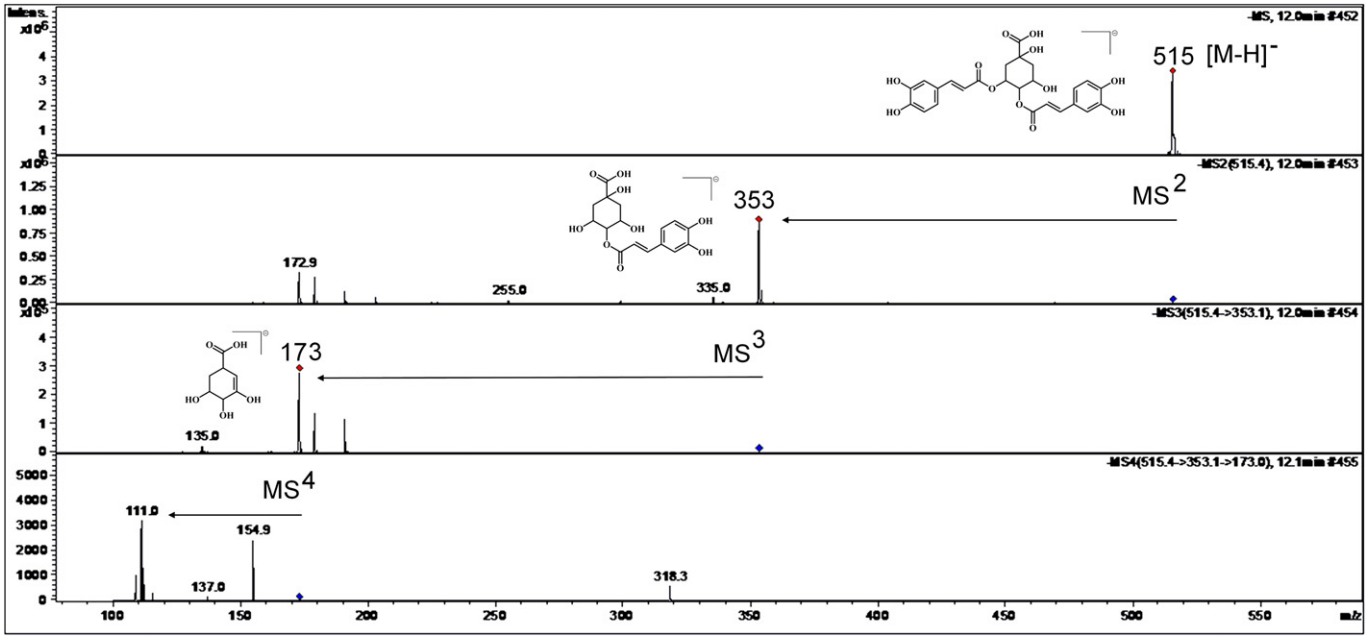


Fig. 2. ESI/MS^n negative mode analysis of 3,4-*O*-diCQA (compound **3**). Sequential fragmentation, MS^n (n up to 4) of the fragment ion at m/z 515.

Table 4
Contents of individual phenolic compounds in *Helichrysum* species from Madeira (mg 100 g^{-1} of dried plant material).

Compound		5-CQA	1,3-diCQA	3,4-diCQA	1,5-diCQA	3,5-diCQA	4,5-diCQA	3,4,5-triCQA	Total amount
H. monizii	Total aerial parts	375.9 ± 0.53	*	16.52 ± 0.045	441.9 ± 0.58	303.29 ± 0.61	31.67 ± 0.056	*	1169.3 ± 1.8
H. melaleucum	Total aerial parts	167.1 ± 0.36	3.91 ± 0.012	3.91 ± 0.013	562.4 ± 0.17	17.64 ± 0.023	50.27 ± 0.22	4.63 ± 0.0032	809.9 ± 0.80
	Leaves	345.0 ± 1.2	5.86 ± 0.031	ND	915.4 ± 1.4	30.61 ± 1.6	60.44 ± 0.43	10.58 ± 0.0076	1367.9 ± 0.64
	Flowers	114.0 ± 0.47	*	ND	628.8 ± 0.11	ND	43.12 ± 0.11	10.15 ± 0.0053	804.03 ± 0.74
	Stems	90.78 ± 0.17	*	ND	78.81 ± 0.034	7.85 ± 0.041	40.48 ± 0.061	ND	210.07 ± 0.27
H. devium	Total aerial parts	35.28 ± 0.56	2.73 ± 0.048	*	125.4 ± 0.028	0.462 ± 0.02	17.33 ± 0.054	5.29 ± 0.0037	186.4 ± 0.71
	Leaves	35.19 ± 0.060	3.29 ± 0.017	1.27 ± 0.0014	88.00 ± 0.079	3.00 ± 0.049	9.45 ± 0.0013	1.929 ± 0.0025	142.1 ± 0.21
	Flowers	129.2 ± 1.2	*	1.89 ± 0.0036	197.3 ± 0.39	49.92 ± 0.19	40.58 ± 0.033	*	422.6 ± 1.8
	Stems	6.21 ± 0.030	1.33 ± 0.018	1.17 ± 0.0163	15.29 ± 0.037	0.44 ± 0.0093	10.13 ± 0.011	*	35.81 ± 0.12
	Roots	27.68 ± 0.0052	18.2 ± 0.052	3.89 ± 0.038	132.2 ± 0.71	60.46 ± 0.070	68.78 ± 0.069	57.59 ± 0.22	368.6 ± 1.2
H. obconicum	Total aerial parts	46.40 ± 0.21	2.15 ± 0.50	18.96 ± 0.25	9.56 ± 0.039	56.53 ± 0.49	29.93 ± 0.19	5.30 ± 0.019	168.8 ± 1.7
	Leaves	52.19 ± 0.73	*	16.99 ± 0.24	83.09 ± 2.9	469.5 ± 0.25	82.53 ± 0.11	*	604.7 ± 1.1
	Stems	65.65 ± 0.12	3.36 ± 0.015	3.46 ± 0.050	34.87 ± 0.19	66.57 ± 0.20	19.88 ± 0.22	*	197.2 ± 0.80

Values are the mean of 3 replicates ± S.D.
*Compounds present in trace amounts (lower than the LOQ);
ND — Not detected.

Due to its rarity, *H. monizzi*, is not used in the traditional medicine to a specific use. Given the high levels of CQAs detected in this plant and its strong antioxidant activity about 10 times the activity of the other 3 subspecies (Gouveia & Castilho, 2011b) *H. monizii* can be a very good natural source for this class of compounds and a program for the reproduction of the plant is now in progress.

4. Conclusions

The HPLC-DAD–ESI/MS^n method developed in this work proved to be reproducible (good selectivity, linearity and precision) and accurate allowing the quantification of 7 caffeoylquinic acids (one monoCQA, five diCQA and one triCQA isomers) present in four endemic *Helichrysum* plants. This was the first time that CQA compounds were quantified in these plants.

The combined use of mass spectrometry enabled the unequivocally identification of these compounds.

The 1,5-*O*-diCQA isomer is the major compound in all plants analyzed with exception of *H. obconicum* where the 3,5-*O*-diCQA is the main compound.

Research is under way to quantify other phenolic compounds, namely flavonoids and malonylcaffeoyl quinic acids, a rarer class of plant components, identified in all the analyzed subspecies of *Helicrhysum*.

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References

Al-Rehaily, A. J., Albishi, O. A., El-Olemy, M. M., & Mossa, J. S. (2008). Flavonoids and terpenoids from *Helichrysum forskahlii*. *Phytochemistry*, *69*, 1910–1914.

Bundy, R., Walker, A. F., Middleton, R. W., Wallis, C., & Simpson, H. C. R. (2008). Artichoke leaf extract (*Cynara scolymus*) reduces plasma cholesterol in otherwise healthy hypercholesterolemic adults: A randomized, double blind placebo controlled trial. *Phytomedicine*, *15*, 668–675.

Carini, M., Aldini, G., Furlanetto, S., Stefani, R., & Facino, R. M. (2001). LC coupled to iontrap MS for the rapid screening and detection of polyphenol antioxidants from *Helichrysum stoechas*. *Journal of Pharmaceutical and Biomedical Analysis*, *24*, 517–526.

Clifford, M. N., Johnston, K. L., Knight, S., & Kuhnert, N. (2003). Hierarchical scheme for LC–MS^n identification of chlorogenic acids. *Journal of Agricultural and Food Chemistry*, *51*, 2900–2911.

Clifford, M. N., Knight, S., & Kuhnert, N. (2005). Discriminating between the six isomers of dicaffeoylquinic acid by LC–MS^n. *Journal of Agricultural and Food Chemistry*, *53*, 3821–3832.

Clifford, M. N., Marks, S., Knight, S., & Kuhnert, N. (2006). Characterization by LC–MS^n of four new classes of *p*-coumaric acid-containing diacyl chlorogenic acids in green coffee beans. *Journal Agricultural Food Chemistry*, *54*, 4095–4101.

Communities, C. o. t. E. (1996). Validation of Analytical Procedures, III: 5626/94. In *International Conference on Harmonization (ICH)*. Brussels.

Coon, J. S. T., & Ernst, E. (2003). Herbs for serum cholesterol reduction — A systematic review. *The Journal of Family Practice*, *52*, 468–478.

Danino, O., Gottlieb, H. E., Grossman, S., & Bergman, M. (2009). Antioxidant activity of 1,3-dicaffeoylquinic acid isolated from *Inula viscosa*. *Food Research International*, *42*, 1273–1280.

Farah, A., Monteiro, M., Donangelo, C. M., & Lafay, S. (2008). Chlorogenic acids from green coffee extract are highly bioavailable in humans. *The Journal of Nutrition*, *138*, 2309–2315.

Gouveia, S. C., & Castilho, P. C. (2009). Analysis of phenolic compounds from different morphological parts of *Helichrysum devium* by liquid chromatography with on-line UV and electrospray ionization mass spectrometric detection. *Rapid Communications in Mass Spectrometry*, *23*, 3939–3953.

Gouveia, S. C., & Castilho, P. C. (2010). Characterization of phenolic compounds in *Helichrysum melaleucum* by high-performance liquid chromatography with on-line ultraviolet and mass spectrometry detection. *Rapid Communications in Mass Spectrometry*, *24*, 1851–1868.

Gouveia, S. C., & Castilho, P. C. (2011). Characterisation of phenolic acid derivatives and flavonoids from different morphological parts of *Helichrysum obconicum* by a RP-HPLC-DAD-(−)-ESI–MS^n method. *Food Chemistry*, *129*, 333–344.

Gouveia, S. C., & Castilho, P. C. (2011a). Antioxidant potential of *Artemisia argentea* L'Hér alcoholic extract and its relation with the phenolic composition. *Food Research International*, *44*, 1620–1631.

Gouveia, S. C., & Castilho, P. C. (2011b). *Helichrysum monizii* Lowe: Phenolic Composition and Antioxidant Potential. *Phytochemical Analysis*, DOI 10.1002/pca.1326.

Gu, R., Dou, G., Wang, J., Dong, J., & Meng, Z. (2007). Simultaneous determination of 1,5-dicaffeoylquinic acid and its active metabolites in human plasma by liquid chromatography–tandem mass spectrometry for pharmacokinetic studies. *Journal of Chromatography B*, *852*, 85–91.

Hu, Z., Chen, D., Dong, L., & Southerland, W. M. (2010). Prediction of the interaction of HIV-1 intergrase and its dicaffeoylquinic acid inhibitor through molecular modeling approach. *Ethnicity & Disease*, *20*, 45–49.

Iwai, K., Kishimoto, N., Kakino, Y., Mochida, K., & Fujita, T. (2004). In vitro antioxidative effects and tyrosinase inhibitory activities of seven hydroxycinnamoyl derivatives in green coffee beans. *Journal of Agricultural and Food Chemistry*, *52*, 4893–4898.

Jaiswal, R., Deshpande, S., & Kuhnert, N. (2011). Profiling the chlorogenic acids of *Rudbeckia hirta*, *Helianthus tuberosus*, *Carlina acaulis* and *Symphyotrichum novae-angliae* leaves by LC–MS^n. *Phytochemical Analysis*, 22, 432–441.

Jaiswal, R., Kiprotich, J., & Kuhnert, N. (2011). Determination of the hydroxycinnamate profile of 12 members of the Asteraceae family. *Phytochemistry*, *72*, 781–790.

Jaiswal, R., Sovdat, T., Vivan, F., & Kuhnert, N. (2010). Profiling and characterization by LC–MS^n of the chlorogenic acids and hydroxycinnamoylshikimate esters in Maté (*Ilex paraguariensis*). *Journal of Agricultural and Food Chemistry*, *58*, 5471–5484.

Jardim, R., & Francisco, D. (2000). *Endemic flora of Madeira.* : Múchia Publicações.

Kim, S. -S., Park, R. -Y., Jeon, H. -J., Kwon, Y. -S., & Chun, W. (2005). Neuroprotective effects of 3,5-dicaffeoylquinic acid on hydrogen peroxide-induced cell death in SH-SY5Y cells. *Phytotherapy Research*, *19*, 243–245.

Krizman, M., Baricevic, D., & Prosek, M. (2007). Determination of phenolic compounds in fennel by HPLC and HPLC–MS using a monolithic reversed-phase column. *Journal of Pharmaceutical and Biomedical Analysis*, *43*, 481–485.

Mabry, T. J., Markham, K. R., & Thomas, M. B. (1970). The ultraviolet spectra of flavones and flavonols, isoflavones, dihydroxyflavonols. *The systematics identification of flavonoids*. New York, NY: Springer-Verlag.

Miketova, P., Schram, K. H., Whitney, J., Kearns, E. H., & Timmermann, B. N. (1999). Mass spectrometry of 3,5- and 4,5-dicaffeoylquinic acids and selected derivatives. *Journal of Mass Spectrometry*, *34*, 1240–1252.

Mondolot, L., La Fisca, P., Buatois, B., Talansier, E., De Kochko, A., & Campa, C. (2006). Evolution in caffeoylquinic acid content and histolocalization during *Coffea canephora* leaf development. *Annals of Botany*, *98*, 33–40.

Ojwang, J. O., Wang, Y. -H., Wyde, P. R., Fischer, N. H., Schuehly, W., Appleman, J. R., Hinds, S., & Shimasaki, C. D. (2005). A novel inhibitor of respiratory syncytial virus isolated from ethnobotanicals. *Antiviral Research*, *68*, 163–172.

Papetti, A., Daglia, M., Aceti, C., Sordelli, B., Spini, V., Carazzone, C., & Gazzani, G. (2008). Hydroxycinnamic acid derivatives occurring in *Cichorium endivia* vegetables. *Journal of Pharmaceutical and Biomedical Analysis*, *48*, 472–476.

Parejo, I., Viladomat, F., Bastida, J., & Codina, C. (2004). Development and validation of a high-performance liquid chromatographic method for the analysis of antioxidative phenolic compounds in fennel using a narrow bore reversed phase C_{18} column. *Analytica Chimica Acta*, *512*, 271–280.

Rivera, D., & Obón, C. (1995). The ethnopharmacology of Madeira and Porto Santo Islands, a review. *Journal of Ethnopharmacology*, *46*, 73–93.

Weisz, G. M., Kammerer, D. R., & Carle, R. (2009). Identification and quantification of phenolic compounds from sunflower (*Helianthus annuus* L.) kernels and shells by HPLC-DAD/ESI–MS^n. *Food Chemistry*, *115*, 758–765.

Zapesochnaya, G. G., Kurkin, V. A., Kudryavtseva, T. V., Karasartov, B. S., Cholponbaev, K. S., Tyukavkina, N. A., & Ruchkin, V. E. (1992). Dicaffeolyquinic acids from *Helichrysum italicum* and *Achillea cartilaginea*. *Chemistry of Natural Compounds*, *28*, 40–44.