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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