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Temperature and density effects of the $scCO_2$extraction of spilanthol from *Spilanthes acmella* flowers

A.M.A. Dias[a], A.C.S. da Silva[b], J.R.S. Botelho[a], R.N.C. Júnior[b], H.C. de Sousa[a,*], M.E.M. Braga[a,*]

[a] *CIEPQPF, Chemical Engineering Department, FCTUC, University of Coimbra, Rua Sílvio Lima, Pólo II – Pinhal de Marrocos, 3030-790 Coimbra, Portugal*
[b] *LABEX (Laboratory of Extraction) – Faculty of Food Engineering, Federal University of Pará, Rua Augusto Corrêa S/N, Guamá 66075-900, Belém, Pará, Brazil*

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ABSTRACT

Spilanthol presents anesthetic, antiseptic and anti-inflammatory capacities, as well as anti-microbial and insecticidal activities. In this work, the supercritical carbon dioxide ($scCO_2$) extraction kinetics of spilanthol from jambu flowers was studied by changing the solvent density (700–900 kg m^{-3}) at different temperatures (323–343 K). Spilanthol was isolated from the obtained extracts by TLC and identified/quantified by GC-FID/MS. The highest global extraction yield (~9%) was obtained at 900 kg m^{-3} and 343 K, while the highest spilanthol yield and selectivity (2.6% and 34.6%, respectively) were obtained at 800 kg m^{-3} and 343 K. Indirect values for the solubility of spilanthol in $scCO_2$ were estimated from the spilanthol extraction kinetic curves and ranged between (0.7–2.6) × 10^{-3} $g_{spilanthol}/L_{CO2(PTN)}$. The high selectivity of the extraction process for spilanthol achieved in the present work indicates potential advantages of using $scCO_2$ to obtain spilanthol from *S. acmella*.

1. Introduction

Despite of their original uses in folk and traditional medicines, natural-origin extracts and/or purified natural products are now being studied for different commercial medical, cosmetic, agricultural and food applications [1].

Plant *N*-alkylamides (NAAs) are important natural-origin bioactive compounds that can be found in more than 25 different plant families [2] and that are particularly abundant in the genus Spilanthes (*Asteraceae* family), such as *S. acmella*, *S. americana*, *S. oppositifolia*, *S. ocymifolia*, *S. ciliate*, *S. calva* and *S. mauritiana*. These plants are widely distributed in tropical and sub-tropical regions of the world [3] and can produce NAAs as secondary metabolites that are known to play important roles in their growth-regulating functions and plant defenses [4]. NAAs chemical structures contain a central amide group (-C(=O)NH-), which is linked to a polyunsaturated aliphatic chain at the carbonyl side and a shorter alkyl chain at the amine side. The large variety of possible substituent chains/groups in both sides of the amide group led to the development of a specific alkylamide's classification system – Alkamide® [5]. This system aims essentially the straightforward identification of NAAs specific structural-functional relationships and therefore to understand the wide variety of biological-pharmacological effects already suggested and/or demonstrated for this family of compounds (which are known to be strongly dependent on their chemical structures).

Therefore, the large number of traditional uses of NAA's producing plants and/or their extracts justifies the current increasing interest in the extraction and isolation of these substances [5]. It has been reported that NAA's present potential applications as analgesics, antioxidants, anti-inflammatories, antibacterials, antifungals, antiparasitics, molluscicides and insecticides as well as tingling and other organoleptic interesting properties for food applications [5,6].

Spilanthol ((2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide) is one of the best known and studied NAAs and represents *ca* 90% of the total NAA's present in *S. acmella* [7]. Nevertheless it can also be found in *Heliopsis longipes* [8,9] in relative high amounts. Spilanthol, spilanthol derivatives or spilanthol-enriched extracts have been screened for their *in vivo* and *in vitro* bioactivities, and namely for their analgesic, anti-inflammatory, anti-oxidant and insectici-

* Corresponding authors.
E-mail addresses: hsousa@eq.uc.pt (H.C. de Sousa), marabraga@eq.uc.pt (M.E.M. Braga).

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dal properties [5,10,11]. Moreover, *in vitro* studies also showed that spilanthol presented a low toxicity to RAW 264.7 cell lines (only a 10% decrease in cell viability at 40 μg/ml) and negligible interactions with other conventional drugs [12]. However and despite of the large number of their potential bioactive properties and applications, to the best of our knowledge there are only a few spilanthol-based commercial products available in the cosmetic and phytotherapic market: buccal gels (Buccaldol® and Indolphar® presenting analgesic and anti-inflammatory activities), tinctures for topical treatment of infections (Vogel spilanthes tincture containing 65% ethanol, from Biohorma, Belgium) and cosmetic creams presenting anti-aging properties (Gatuline® Expression, from Gattefossé, France; *Acmella* Pur Active, from Etat Pur, France and Human + Kind Anti-aging, from Ireland). In addition, there are also some reports, including industrial patents, claiming the use of NAAs (including spilanthol) as taste and flavor enhancers in food, drinks and medicines [13,14–16].

One of the main reasons that may be hampering more widespread uses of spilanthol and/or spilanthol-enriched extracts may be the lack of unequivocal chemical composition-bioactivity data, since most of the studies reported so far in the literature generally employ complex and non-purified plant extracts to perform the *in vitro* and *in vivo* bioactivity studies. Moreover, and as reviewed and summarized in Table 1, different extraction methodologies, using different solvents and/or experimental conditions, and/or parts of the plant (and from different origins), have been used so far to obtain purified NAAs or extracts from *S. acmella.* This makes quite difficult any attempts of comparison in terms of extraction yields, of accurate chemical composition characterization, as well as the systematic analysis of the *in-vitro* and *in-vivo* tests/results, and thus of the precise knowledge of the benefits and potentialities of these substances/extracts for pharmacological, biomedical and food/cosmetic applications.

As can be seen in Table 1, spilanthol can be extracted from *S. acmella* using both polar (methanol, ethanol, water) and apolar ($scCO_2$ or hexane) solvents because of its slight-amphiphilic nature that results from the conjugation of the relatively polar amide and a less polar poly-unsaturated alkyl chain. In a previous work, our research team found out that $scCO_2$ extraction was an efficient method to extract spilanthol from jambu flowers, yielding spilanthol-enriched and solvent-free extracts, and that may be adequate to be used without other further complex, time consuming and solvent-dependent purification processes [27]. Moreover, it was also concluded that the spilanthol yields obtained from flowers were significantly higher than those recovered from *S. acmella* leaves and stems, which justifies the particular interest in studying this part of the plant (and as also evidenced in Table 1 by the work of other researchers).

The enhanced solubility of spilanthol in $scCO_2$ is probably due to the non-polar character of the poly-unsaturated aliphatic chain at the carbonyl side of the molecule as well as to the resonance characteristics of the amide bonds which originate large dipole moments and therefore favorable CO_2-spilanthol interactions. Azofra and co-authors recently showed that amide derivatives have significant affinity for carbon dioxide through the formation of three different types of stable amide-CO_2 complexes which result from its simultaneous and cooperative Lewis acid and Lewis base behaviors [36].

Therefore, the main objective of this work was to study and to optimize the $scCO_2$ extraction yields and selectivity for the extraction of spilanthol from *S. acmella* flowers by using different extraction conditions. For this purpose, the extraction kinetic curves were obtained at different $scCO_2$ densities (700, 800 and 900 kg m^{-3}) and at different extraction temperatures (323, 333 and 343 K). A response surface analysis methodology was employed to evaluate the obtained results.

2. Materials and methods

2.1. Raw material and chemicals

Spilanthes acmella samples were obtained in Belém, Pará, Brazil. The specie was identified as the same that was used in a previous work [27] (voucher MG200834, deposited at the Emílio Goeldi Paraense Museum, Belém, Brazil). Raw material was dried at room temperature and plant flowers were separated and comminuted using a knife mill (Tecnal – TE 650, Brazil). Particle size distribution was obtained using a Tyler sieve series, mesh 24–48 (W.S. Tyler, USA). The humidity of the samples was determined by the Jacobs xylol distillation method [37] in duplicate. Raw material was stored under vacuum, at 283 K and away from light, before the extraction experiments.

Carbon dioxide (99.9%, Gases Gama, Brazil), ethyl acetate (99.9%, Chromasolv Plus, Sigma-Aldrich), *n*-hexane (96%, Merck) and xylol (p.a., Ecibra, Brazil) were obtained and used without further purification. Thin Layer Chromatography (TLC) analyses were performed using silica gel plates (20 × 20 cm, thickness 0.2 mm, Fluka, Germany). Chemicals used to prepare the Dragendorff's reagent (potassium bismuth nitrate (98%), acetic acid (99.7%) and potassium iodide (99%)) were obtained from Sigma-Aldrich.

2.2. Experimental $scCO_2$ extraction procedures

Supercritical carbon dioxide extractions were performed using a SPE-ED SFE system (Applied Separations, model 7071, Allentown, USA), consisting of a high pressure extractor installed in a controlled temperature oven and of a booster pump to deliver the pre-liquefied CO_2. The high pressure extraction cell (internal volume is $\sim 1.05 \times 10^{-4}$ m^3) was filled with pre-processed plant flowers (~ 12 g). Bed apparent density was 133 kg m^{-3}. The outlet CO_2 mass flow was maintained at 7.4×10^{-5} kg s^{-1} and measured at PTN using a mass flow meter (Alicat Scientific M 5SLPM, EUA). Experiments were performed at three different $scCO_2$ densities (~700, ~800 and ~900 kg m^{-3}) and at three temperatures (323, 333 and 343 K). Operating densities were achieved by changing extraction pressure according to the following conditions: at 323 K (15, 22 and 35 MPa); at 333 K (19, 27 and 42 MPa) and at 343 K (22, 32 and 49 MPa). Extracts were collected into refrigerated flasks at predefined time intervals and during a 3 h extraction period. The dynamic period was preceded by a static period of 30 min to allow an initial contact of the raw material with the extraction solvent. Collected extracts were kept away from light, at approximately 255 K, until further analyses.

2.3. Isolation and identification of spilanthol by TLC

Spilanthol was isolated from a TLC plate which was previously prepared with an aliquot of the extract obtained at 323 K and 25 MPa. TLC was performed using hexane/ethyl acetate (2:1, v/v) as the mobile phase and following a previously reported procedure [27]. The sample corresponding to R_f 0.5 was dark under UV light and brown after being revealed using the Dragendorff reagent, thus confirming the presence of an alkylamide rich fraction [27,38].This isolated fraction was used as the spilanthol standard (as confirmed by GC–MS analysis) and to obtain the GC calibration curve (to calculate spilanthol extraction yields).

2.4. Gas chromatography (GC–MS and GC-FID)

The identification of spilanthol was performed by GC–MS (Agilent Technologies, USA), model GC 7890A coupled to a G3171A MSD mass selective detector, using a silica capillary column (30 m × 0.32 mm i.d.; df, 0.25 μm) covered with 5% phenyl/95%