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Research paper

Determination of coumarins in the roots of *Angelica dahurica* by supercritical fluid chromatography

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ABSTRACT

The fact that supercritical fluid chromatography (SFC) offers many desirable features is known for a long time. Yet, the number of applications on natural products is still limited, because robust and user-friendly instrumentation became available just a few years ago. As coumarins hardly have been studied by this technique we developed the first SFC assay for their determination in crude plant material. After method optimization eight standard compounds, including simple coumarins, linear and angular furanocoumarins, could be baseline separated in 6 min using an Acquity UPC^2 CSH Fluoro-Phenyl 1.7 μm column with supercritical CO_2, methanol and diethylamine as mobile phase. Method validation confirmed that the assay is linear ($R^2 \geq 0.9995$), precise (intra-day variation ≤ 5.8%; inter-day variation ≤ 4.4%) and accurate (recovery rates from 96.5 to 104.2%). Detection limits determined at 300 nm were below 2 ng on-column, and the method showed to be well suited for the analysis of coumarins in *Angelica dahurica* roots. It was observed that qualitative as well as quantitative composition vary significantly. In all samples Imperatorin (0.09–0.28%) was the major coumarin, followed either by Isoimperatorin or Oxypeucedanin; the total coumarin content ranged from 0.16 to 0.77%. The results were in good agreement to published data, so that because of its speed and green nature SFC is definitely an interesting alternative for the analysis of this important class of natural products.

1. Introduction

Supercritical fluids, which are obtained when a substance is maintained above its critical temperature and pressure, are often considered as ideal mobile phases for chromatography [1]. With combined features of the gaseous and liquid state (e.g. high diffusivity and low viscosity) they enable fast and efficient separations [2]. Carbon dioxide based fluids are used almost exclusively today, because with critical values of 31 °C and 74 bar the supercritical state is easily reached. Additionally the compound is inert, non-flammable and cheap [3]. As it converts to a gas under normal pressure conditions it is convenient for isolation purposes, and often termed as "green" and environmental friendly alternative to the use of organic solvents. Even if the technique was called HPGC or DGC then, the option of using a supercritical fluid for separation purposes has been known for more than 50 years [4]. Yet, just recently reliable instruments and specific stationary phases became available, so that SFC gained momentum and increased attention. For natural products analysis SFC still has to be considered as an "exotic" alternative. There are a few applications on vitamins, fatty acids, terpenes, alkaloids and flavonoids, but many relevant compound classes never have been investigated [1]; for example, just recently we reported on the first SFC method for anthraquinones [5].

Coumarins are naturally occurring 1,2-benzopyrone derivatives, representing the active principles in several important medicinal plants. One of the most relevant among them is *Angelica dahurica*, for which monographs are found in the current European and Chinese Pharmacopeia. The roots of the plant are widely used in Traditional Chinese Medicine for the treatment of migraine, common cold and swellings [6]. Pharmacological data support these indications, as coumarins like Imperatorin and Oxypeucedanin are antioxidants [7], moderate TRPV1 (a channel implicated in detecting noxious stimuli) agonists [8], and anticonvulsants [9]. The preferred technique for the analysis of coumarins is HPLC [10–12]; yet, GC [13], CE [14], and CEC [15] are other suitable options. SFE was employed for the preparation of *Angelica* extracts [16,17], and preparative SFC utilized for isolation of the single compounds

Abbreviations: ABPR, automated backpressure regulator; DGC, dense gas chromatography; HPGC, high pressure gas chromatography; SFC, supercritical fluid chromatography; SFE, supercritical fluid extraction; TCM, traditional Chinese medicine; TRPV1, transient receptor potential vanilloid type 1.

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[18–20]. Additionally, coumarins were used in methodological SFC studies as standard compounds [21,22]. Desmortreux and colleagues reported on the analysis of furocoumarins in essential oils (lemon) by SFC [23], yet what has never been described is the qualitative or quantitative analysis of coumarins in crude plant material.

2. Materials and methods

2.1. Materials

Standards of eight coumarins (Angelicin (**1**), Osthole (**2**), Imperatorin (**3**), Xanthotoxin (**4**), Isoimperatorin (**5**), Oxypeucedanin (**6**), Xanthotoxol (**7**), and Umbelliferone (**8**); for structures see Fig. 1) were purchased from PhytoLab (Vestenbergsreuth, Germany). They had a purity of ≥98.8%. The plant material was purchased 2015 in local pharmacies in Innsbruck, Austria, or obtained from wholesale merchants in Austria and Germany; voucher specimens (Anda-15-1 to Anda-15-5) are deposited at the Institute of Pharmacy, Pharmacognosy, at the University of Innsbruck.

For SFC analysis the mobile phase comprised 4.5 grade carbon dioxide (purity ≥ 99.995%; Messer, Gumpoldskirchen, Austria) and methanol with diethylamine. All solvents/chemicals used for extraction and analysis were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.2. Sample preparation

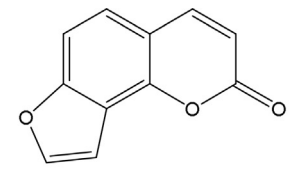
The finely powdered plant material (root, 500 mg) was extracted with 2.5 ml methanol by sonication for 20 min. Then the sample was centrifuged for 3 min at 3000 RPM, and the clear supernatant placed in a 10 ml volumetric flask. This extraction procedure was repeated three more times, the extracts were combined, and the flask filled to volume with extraction solvent. Prior to analysis of the samples, which are stable for at least two weeks if stored at 4 °C, the solutions were membrane filtered (Minisart SRP 15, 0.2 μm, PTFE-membrane; Sartorius, Göttingen, Germany). Due do the high content of **6** the sample solutions of Anda-15-2 and Anda-15-5 had to be diluted with methanol in the ratio 1:5 before being quantified by SFC.

2.3. Analytical method

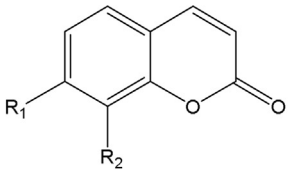
For all experiments an Acquity UPC^2 SFC instrument from Waters (Milford, MA, USA), equipped with convergence-, sample-, binary solvent- and column manager, and PDA detector, was used. The operating software was Empower 3, release 2 (Waters). Optimum separations were obtained on an Acquity UPC^2 CSH Fluoro-Phenyl 1.7 μm column (3.0 × 100 mm; Waters), and a mobile phase comprising CO_2 (A) and 0.1% diethylamine in methanol (B). The applied gradient was as follows: from 100A/0B in 5 min to 97A/3B, in 2 min to 90A/10B, and held at this composition for 1 min (total runtime: 8 min); then the column was equilibrated for 3 min under the initial conditions. Flow rate, column temperature and detection wavelength were set to 1.5 ml/min, 30 °C and 300 nm. The injected sample volume was 1 μl and the ABPR adjusted to 130 bar.

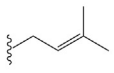
2.4. Validation

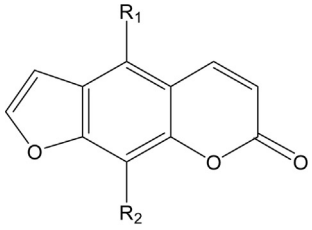
The performance characteristics of the developed SFC procedure were studied in validation experiments. For establishing calibration curves and the determination of linearity approx. three mg of each standard were, accurately weighted, dissolved in 10.00 ml methanol. This stock solution was serially diluted with the same solvent in the ratio 1:2 to prepare calibration solutions and determine LOD and LOQ. The latter two were visually evaluated representing standard concentrations equivalent to signal-to-noise ratios of 3 and 10, respectively. Selectivity was concluded by evaluation of the PDA data, i.e. utilizing the peak purity option in the software. Short term precision (repeatability) was deduced from relative standard deviations well below 5% when analyzing the same sample solutions several times. Intermediate precision



Angelicin (**1**)



	R_1	R_2
Osthole (**2**)	OCH_3	
Umbelliferone (**8**)	OH	H



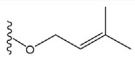
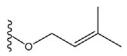
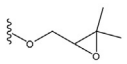
	R_1	R_2
Imperatorin (**3**)	H	
Xanthotoxin (**4**)	H	OCH_3
Isoimperatorin (**5**)		H
Oxypeucedanin (**6**)		H
Xanthotoxol (**7**)	H	OH

Fig. 1. Chemical structures of the determined coumarins.