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Two-dimensional multi-heart cutting centrifugal partition chromatography–liquid chromatography for the preparative isolation of antioxidants from Edelweiss plant☆

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ABSTRACT

The Edelweiss plant has been recognized as a very valuable source of anti-aging principles due to its composition of antioxidants compounds: leontopodic acid A and 3,5-dicaffeoylquinic acid. In this work, off-line multi-heart cutting CPC-LC separation was set up at industrial scale in order to isolate and produce new high quality reference material of these two antioxidants from Edelweiss. For this purpose, CPC and HPLC methods were developed and optimized at laboratory scale and a comprehensive CPCxHPLC analysis of the crude extract was established. Thereby, the CPC method led to a first separation of the target compounds according to their partition coefficient in the solvent system and the HPLC method was performed on the recovered fractions to lead to a second separation. A 2D CPCxHPLC plot was established in order to know the fractions to select at the industrial scale. Then, the CPC and HPLC methods were transferred at industrial scale and the multi-heart cutting CPC-LC was performed in off-line mode. Using CPC with methyl ter-butyl ether-water 1:1 (v/v) solvent system and LC with Denali C18 column, 2 g of crude extract sample were injected and leontopodic acid A and 3,5-dicaffeoylquinic acid were recovered with purity over 97%. The compounds were identified by MS and NMR.

1. Introduction

Leontopodium alpinium, commonly known as Edelweiss, is one of the most famous plants of the European Alps. In folk medicine, extracts of Edelweiss are used for the therapy of abdominal aches, angina, bronchitis, cancer, diarrhea, dysentery and fever for humans as well as for livestock [1,2]. Indeed, this plant shows a wide diversity of secondary plant metabolites such as phenolic acids, lignans, flavonoids, sesquiterpenes, coumarins, benzofuran and others [3–5]. Wild Edelweiss is protected by the law but the plant is now cultivated in Switzerland and extracts of the aerial parts are used for their anti-oxidative properties [1,3,6]. From the early 2010′s, the Edelweiss plant extracts have been recognized as a very valuable source of skin anti-aging principles by the cosmetic industry and the main actives substances are phenylpropanoids such as leontopodic acids [7,8].

In order to improve clinical research and provide new high quality standards, the production of two antioxidants from Edelweiss plant is required, namely: leontopodic acid A and 3,5-dicaffeoylquinic acid. In recent years, Schwaiger et al. [9] and Ganzera et al. [10] developed different chromatographic methods for the identification and the quantification of major phenolic Edelweiss constituents such as high performance liquid chromatography and micellar electrokinetic capillary chromatography. The analysis exhibit the complexity of these plant extracts. However, currently no preparative assay of these phenylpropanoids is available to isolate and produce the compounds of interest. As the plant extract is complex, the isolation of compounds using preparative HPLC cannot be considered.

For the isolation and production of phenolic compounds and phenylpropanoids from natural products, Countercurrent Chromatography (CCC) is widely used [11–14]. The countercurrent chromatography is a liquid chromatography technique that uses two immiscible liquid phases without any solid support. This tech-

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nique eliminates irreversible adsorptive loss of samples onto the solid support matrix used in conventional chromatography and has the advantage to be versatile due to the huge choice of solvent combinations. With a loadable stationary phase, CCC is a very interesting preparative separation technique [15].

The purpose of the work is the purification and isolation of multiple targets from complex natural product. For this purpose, a complementarity of techniques was developed in order to combine two different selectivities through two different mechanisms of separation. A two-dimensional (2D) chromatography methodology coupling independent separation techniques provides higher peak capacity, resolution and selectivity. The countercurrent chromatography technique is set to be the first dimensional separation due to the loadable stationary phase. The HPLC technique, more efficient, is used as second dimensional separation. Several preparative applications using this strategy was reported in these recent years, especially in natural products field. The hyphenation can be on-line and comprehensive [16] with flow programming CCC [17], on-line and heart-cutting [18,19] with stop-and-go CCC [20] or off-line and heart-cutting [21].

In the present study, an off-line multi-heart-cutting hyphenation of countercurrent chromatography using Centrifugal Partition Chromatography device (CPC) and preparative liquid chromatography was established in order to produce the two antioxidants from the Edelweiss extract. The CPC and HPLC methods were first developed separately at laboratory scale. Then the 2D hyphenation CPCxLC was performed off-line in a comprehensive mode at laboratory scale. Finally, the methods were transferred at industrial scale for the production of the two antioxidants by multi-heart-cutting CPC-LC meaning that only the fractions of interest were sent from the CPC to the second LC dimension.

2. Experimental

2.1. Chemicals and materials

All solvents used for the preparation of the sample, the HPLC analysis and the CPC separation were of analytical grade. Methyl ter-butyl ether was purchased from Acros Organics (Fisher Scientific, Illkirch, France). HPLC grade solvents for HPLC were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

The extract of the aerial part of Edelweiss was provided by the company Extrasynthese (Genay, France). It had to be stored away from light at −20 °C in a freezer to avoid any degradation. Leontopodic acid A and 3,5-dicaffeoylquinic acid authentic samples were provided by Prof. Schwaiger (Innsbruck University, Austria).

2.2. Instrumentation

All instruments used in this study are commercially available.

The CPC instrument employed at the laboratory scale was the FCPC-C from Kromaton Rousselet-Robatel (Annonay, France) with interchangeable columns (or rotors). The column had an exact volume of 34.5 ml. The apparatus was equipped with a Shimadzu preparative pump LC-20AP (Noisiel, France), a Shimadzu UV/VIS detector SPD-20A set up at 220 nm, a manual sample injection valve with a 350 μl sample loop and a fraction collector. The rotor was thermostated at 21 °C. The data were collected with Azur software provided by Datalys (Le Touvet, France).

The CPC instrument employed at industrial scale was the SCPC-1000 from Armen Instrument (Saint-Avé, France). The column had an exact volume of 1.088 liters. The apparatus was equipped with a Spot Prep II integrated system from Armen Instrument. This equipment is the assembly of a quaternary pump, an automatic sample injection valve fitted on a 20 ml sample loop, a UV/VIS spectrophotometer dual wavelength set up at 220 nm and 330 nm and a fraction collector. The Armen Glider Prep software installed in the integrated computer allowed the control of the apparatus and the data acquisition.

The HPLC system used at laboratory scale as second dimension was an Alliance 2690 system from Waters (Saint-Quentin-en-Yvelines, France) using a binary solvent delivery system, an autosampler and a Photodiode Array detector Waters 996 set up at 330 nm. A reversed-phase Grace Vydac Denali C18 column (150 mm × 4.6 mm i.d., 5 μm) was used as the second dimensional stationary phase. The data acquisition was performed by EmPower software from Waters.

The HPLC system used at preparative scale was composed of a Reveleris integrated system for injection, detection and collection, set up with 20 ml sample loop and a wavelength of 330 nm. A reversed-phase Grace Vydac Denali C18 column (300 mm × 50 mm i.d., 10 μm) was used as the stationary phase.

All the analysis for the methods development and the fractions control were performed using an Acquity Ultra Performance Liquid Chromatography system from Waters. The system was equipped with a binary solvent delivery system, an autosampler and a Photodiode Array detector set up at 330 nm. The data acquisition was performed by EmPower software from Waters. A reversed-phase Acquity UPLC CSH Phenyl-Hexyl column (100 mm × 2.1 mm i.d., 1.7 μm) was used as stationary phase.

2.3. Selection of CPC solvent system

The solvent system was selected according to the partition coefficient (K_D) of each target component to separate: leontopodic acid A et 3,5-dicaffeoylquinic acid. The K_D value was measured by HPLC analysis using the Shake-Flask methodology. A suitable amount of crude extract was added to a test tube and 2 ml of each of the equilibrated two-phase solvents was added. The tube was shaken vigorously to equilibrate the compounds between the two phases. After partition equilibrium the upper and lower phases were separately taken into vials and 20 μl of each phase was analyzed by HPLC-UV. The peak area of the target compound in the upper phase was recorded as A_{upper} and the one in the lower phase as A_{lower}. The K_D value was calculated according to the following equation: $K_D = A_{upper}/A_{lower}$.

2.4. Preparation of two-phase solvent system and sample solutions

A two-phase solvent system composed of methyl ter-butyl ether-water pH 3 at a ratio of 1:1 (v/v) was developed. At the laboratory, according to the selected ratio, 1000 ml solvent system was thoroughly equilibrated in 1 l bottle at room temperature for 10 min. At industrial scale, 8 l of solvent system was prepared in a 10 l bottle and directly used in CPC.

The sample solution for UHPLC analysis of crude extract was prepared by dissolving 10 mg of crude extract in 1 ml of water-acetonitrile 1:1 (v/v).

For the CPCxLC method development at laboratory scale, 1, 2, 20 and 50 mg of crude extract was dissolved in 1 ml of methyl ter-butyl ether. The solutions were vortexed for 4 min then sonicated for 20 min and centrifuged for 20 min at 4000 rpm. The supernatant was taken and 350 μl was injected. Finally, for the developed CPCxLC hyphenation at laboratory scale, the sample solution was prepared by dissolving 50 mg of crude extract in 1 ml of methyl ter-butyl ether. For the HPLC method development at laboratory scale, 10 mg of crude extract was dissolved in 2 ml of water-acetonitrile 80:20 (v/v) and then filtrated.

For the CPC-LC hyphenation at industrial scale, the sample solution was prepared by dissolving 2 g of crude extract into 40 ml of