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Application of preparative high-speed counter-current chromatography/electrospray ionization mass spectrometry for a fast screening and fractionation of polyphenols

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Abstract

This study presents the novel direct coupling of preparative high-speed counter-current chromatography with electrospray ionization mass spectrometry (HSCCC/ESI-MS–MS) for continuous MS data acquisition, and '*target*'-guided fractionation of polyphenolic compounds from sea buckthorn juice concentrate (*Hippophaë rhamnoides* L. ssp. *rhamnoides*, Elaeagnaceae). It was demonstrated that a combination of HSCCC and ESI-MS–MS is a versatile tool for fast screening and sensitive detection of bioactive natural products in crude extracts. Sample fractionation will be simplified by knowing the continuous MS data of the complete HSCCC separation. Already during the separation, immediate structural information of the separated compounds is provided. Peak purity of HSCCC is assessable by complete ion traces and fractions can directly be evaporated for further one-/two-dimensional NMR analysis.
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1. Introduction

In the field of phytochemical analysis of food and medicinal plants, rapid detection methodologies for '*target*'-oriented screening are required with respect to detecting potentially biological active natural products. Various modern '*on-line*' coupling techniques have already been implemented in routine analysis, such as liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS), and LC/atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS). These methods deliver immediate structural information. Most of the existing procedures work on analytical scale in order to evaluate the occurrence of interesting lead-structures. In a new approach our study utilized the direct coupling of high-speed counter-current chromatography with electrospray ionization mass spectrometry (HSCCC/ESI-MS–MS) to generate continuous molecular mass data with MS–MS fragmentation pattern and simultaneously fractionation of crude extracts on preparative scale. HSCCC/ESI-MS–MS enabled the on-line monitoring of polyphenols in sea buckthorn juice concentrate (*Hippophaë rhamnoides* L. ssp. *rhamnoides*) and the observation of concentration-dependent retention time shifts for protocatechuic acid.

The well-known advantages of HSCCC are the support-free all liquid–liquid chromatography separations that are increasingly applied for preparative isolation of thermal- and light-sensitive bioactive natural products [1]. Earlier experimental trials were solely focussed on analytical high-speed counter-current chromatography (HSACCC)/MS operated with various ionization techniques [2–4].

In this study we will demonstrate that combining the preparative capacities of HSCCC with the low detection limits of ESI-MS–MS is a versatile tool for '*target*'-oriented and rapid

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compound screening in isolation procedures of natural products from food and medicinal plant extracts.

2. Experimental

2.1. Reagents

Organic solvents, dichloromethane and ethyl acetate used for the preparation of sea buckthorn juice concentrate fractions for HSCCC were of analytical grade (Merck, Darmstadt, Germany). For HSCCC separations with ESI-MS–MS detection, HPLC grade *n*-hexane, *n*-butanol (Merck, Darmstadt, Germany) and Nanopure (Barnstead, USA) water were used.

2.2. Processing of sea buckthorn juice concentrate

The sea buckthorn fruits used in this study were harvested in Romania in September 2005. The frozen berries were pre-heated (8–12 °C) before mashing. The mash was subjected to pectolytic enzymes treatment (1–2 h on 52 °C) and separated into juice and pomace by a decanter machine. The turbid juice product, highly concentrated in pulp and oil was clarified by a plate separator. Further, sea buckthorn juice was clarified with fining agents, i.e. bentonite (8–12 h, 10–12 °C). After filtration with diatomeous earth under vacuum, the clear juice was concentrated by thermovacuum evaporation (five stage evaporator, 80–85 °C). The Brix value was 65 for clear juice concentrates. Before aseptic filling the juice concentrate was treated in a HTST process (high-temperature-short-time treatment: 90 °C, 45 s) and rechilled, immediately.

2.3. High-speed counter-current chromatography (HSCCC)

The preparative HSCCC instrument used in the present study was a multilayer coil planet centrifuge model CCC 1000 (Pharma-Tech Research Corp., USA), equipped with three preparative coils connected in series (polytetrafluorethylene (PTFE) tubing: 165 m × 2.6 mm i.d., 850 mL total volume). A manual sample injection valve with a 25 mL loop was used to introduce the sample into the coil system. The mobile phase was delivered with a Biotronik BT 3020 HPLC pump (Jasco, Grossumstadt, Germany).

2.4. Preparation of Hippophaë juice concentrate fractions for HSCCC

For removing lipophilic and amphiphilic natural products of emulsifying capacities, such as sterols, fatty acids, and phospholipids, the concentrated juice of *H. rhamnoides* L. ssp. *rhamnoides* (1015 g, Bayernwald Früchteverwertung, Germany) was exhaustively defatted with *n*-hexane (total volume: 2000 mL). In the subsequent step, similar solvent treatment was carried out with dichloromethane. The polar residue was extracted six times with ethyl acetate (500 mL). After filtration, the ethyl acetate extract was evaporated to dryness by rotary evaporation at 40 °C and 40 mbar to yield 31.6 g of a crude extract enriched in flavonoids. This extract was used for subsequent preparative HSCCC.

2.5. Preparation of the two-phase solvent system and sample solutions for HSCCC

The HSCCC experiments (A and B, cf. Section 2.6) were performed with a two-phase solvent system composed of *n*-hexane–*n*-butanol–water (1:1:2, v/v/v) which is characterized by excellent retention capabilities. After thorough equilibration of the solvents in a separatory funnel at ambient temperature, the two phases were separated shortly before use and degassed by ultrasonication. The upper organic phase was used as stationary phase and the lower aqueous phase as mobile phase using the HSCCC instrument in the '*head-to-tail*' mode.

2.6. Separation procedure of HSCCC

Initially, the triple-coil column system was completely filled with the upper organic phase. After starting the rotation of the system (800 rpm), the sample solution (experiment A: 10 mg; experiment B: 500 mg) was introduced in a mixture of 14 mL consisting of upper and lower phase (1:1, v/v) into the separation column through an injection loop. The lower phase was pumped into the '*head*'-end of the HSCCC coil column at a flow rate of 3 mL min^{-1}. The effluent stream leaving from the '*tail*'-outlet of the coil-column was transferred and divided by a Y-splitter (experiment A) or a T-splitter unit (experiment B) for MS-detection and the sampling in a fraction collector (Fig. 1). After completing the separation, the solvent in the coil column was ejected with nitrogen gas. Volumetric measurement showed retention of 90% of stationary phase solvent. The applied biphasic solvent system was stable and almost no '*carry-over*' of

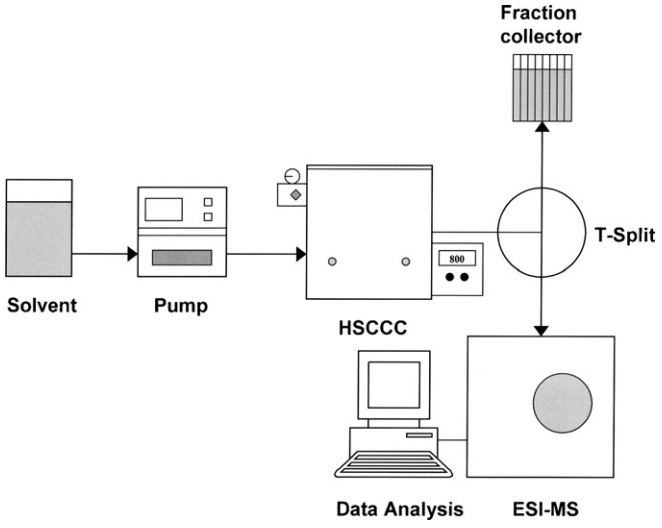

Fig. 1. Schematic diagram of the preparative HSCCC/ESI-MS–MS system. The preparative HSCCC instrument was a multilayer coil planet centrifuge model CCC 1000 (Pharma-Tech Research Corp., USA) coupled to a Bruker Esquire LC–MS ion trap multiple mass spectrometer (Bruker Daltoniks, Bremen, Germany) in negative ionization mode analyzing ions from *m/z* 50 up to *m/z* 1500 amu.

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