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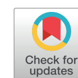
Multiple injection mode with or without repeated sample injections: Strategies to enhance productivity in countercurrent chromatography



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ABSTRACT

Countercurrent chromatography (CCC) is an all liquid based separation technique typically used for the isolation and purification of natural compounds. The simplicity of the method makes it easy to scale up CCC separations from analytical to preparative and even industrial scale. However, scale-up of CCC separations requires two different instruments with varying coil dimensions. Here we developed two variants of the CCC multiple injection mode as an alternative to increase the throughput and enhance productivity of a CCC separation when using only one instrument. The concept is based on the parallel injection of samples at different points in the CCC column system and the simultaneous separation using one pump only. The wiring of the CCC setup was modified by the insertion of a 6-port selection valve, multiple T-pieces and sample loops. Furthermore, the introduction of storage sample loops enabled the CCC system to be used with repeated injection cycles. Setup and advantages of both multiple injection modes were shown by the isolation of the furan fatty acid 11-(3,4-dimethyl-5-pentylfuran-2-yl)-undecanoic acid (11D5-EE) from an ethyl ester oil rich in 4,7,10,13,16,19-docosahexaenoic acid (DHA-EE). 11D5-EE was enriched in one step from 1.9% to 99% purity. The solvent consumption per isolated amount of analyte could be reduced by ~40% compared to increased throughput CCC and by ~5% in the repeated multiple injection mode which also facilitated the isolation of the major compound (DHA-EE) in the sample.

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1. Introduction

Countercurrent chromatography (CCC) is a versatile technique for the separation and purification of natural compounds which is solely based on their distribution coefficient in two immiscible phases of a solvent system [1–3]. High purity compounds isolated with CCC may be used in biochemical studies or as analytical quantification standards [4–6]. In either case the availability of higher amounts is beneficial. The absence of a solid stationary phase makes it easy to increase the throughput of a separation by the scale-up of the column dimensions from analytical to preparative or even industrial scale [7–9]. Within the scale-up process, productivity is increased by separating greater sample amounts in the same time with identical purities of the compounds [10]. In this context, a linear correlation was found between flow rate and coil volume and an efficient scale-up could be achieved by increasing both, the tubing bore and the length of the column [10,11]. For instance, Wood et al. [10] succeeded in the scale-up of the CCC separation of benzyl alcohol and *p*-cresol from an analytical CCC instrument (5.4 mL coil volume) to a preparative CCC instrument (4600 mL coil volume) by increasing the flow rate at the same volumetric scale-up factor from 1 to 850 mL/min. However, the scale-up process typically requires two instruments with different coil dimensions.

In this study we investigated opportunities to enhance productivity (i.e. saving time and solvent consumption) in natural product isolation with only one CCC instrument. As part of this concept we wished to reduce solvent consumption by omitting high flow rates of the mobile phase. On the one hand this is desirable in terms of economy and environmental friendliness [12,13]. On the other hand, higher flow rates lead to higher internal pressures which – if too high – may cause leakages in pressure sensitive parts of the instrument and are therefore not always tolerated by the system. Solvent consumption was reduced by the introduction of a CCC elution mode in which the sample is introduced at different positions into the CCC system and simultaneously developed and separated. The so-called multiple injection mode requires only one pump for the transport of mobile phase and was implemented by the introduction of a 6-port selection valve, T-pieces and sample

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loops. In addition, we aimed to transfer the method into repeated injection operation. Method development, evaluation and comparison of the multiple injection mode with other methods was combined with the isolation of the ethyl ester of 11-(3,4-dimethyl-5-pentylfuran-2-yl)-undecanoic acid (11D5-EE) which is the most widely distributed furan fatty acid in food [14]. Furan fatty acids are characterized by a mono- or dimethyl-substituted furan moiety in the center of the alkyl chain and are considered to be valuable food ingredients and very effective antioxidants [15–17]. Due to their high radical scavenging activity furan fatty acids were discussed to be the reason for the cardioprotective effect of a diet rich in fish oil [17]. However, standards of furan fatty acids are currently not commercially available so that research is limited in the field [18]. Contribution of 11D5 to the fatty acid pattern in food is low and typically <1% [17,19]. Yet, screening of different raw materials pointed us to a commercial omega-3 product generated from fish oil, which was comparably rich in 11D5 and which only contained a few fatty acids as ethyl esters. This sample was used for various CCC separations.

2. Materials and methods

2.1. Organic solvents and chemicals

Acetonitrile (>99.9%) and *n*-hexane (>95%) were from Th. Geyer (Renningen, Germany). Ethanol (distilled before use) and sulfuric acid were from Carl Roth (Karlsruhe, Germany). Methanol (>99.8%) was from Sigma-Aldrich (Taufkirchen, Germany) and myristic acid (14:0; >99%) was from Merck Chemicals (Darmstadt, Germany).

2.2. Samples and standards

Different fish oil samples, capsules and omega-3 oils were initially tested on the presence of furan fatty acids. A product called “DHA ethyl ester oil” from the research and development center of KD Pharma (Bexbach, Germany), which is currently not commercially available, but similar to other products marketed, was finally selected. This product was best suited for the isolation because it contained a reasonable share of 11D5 ethyl ester (11D5-EE) together with only a few other ethyl esters of (omega-3) fatty acids. Both circumstances indicated that it would be a good source for the isolation of 11D5-EE. The internal standard 14:0-EE, used to level out differences in the GC injection volume, was prepared from 10 mg 14:0 using the protocol of Wendlinger et al. [14] with ethanol instead of methanol.

2.3. Gas chromatography with mass spectrometry (GC/MS)

GC/MS measurements were performed with a 6890/5973 GC/MS system (Agilent Technologies, Santa Clara, CA), equipped with an MPS 2 autosampler (Gerstel, Mülheim a.d. Ruhr, Germany) and a 30 m x 0.25 mm i.d., 0.25 μm film thickness HP-5MS (95% methyl, 5% phenyl polysiloxane) capillary column (Agilent, Waldbronn, Germany) using parameters recently described in details [20]. All measurements were carried out in full scan mode (*m*/*z* 60–600). The oven program started with an initial temperature of 60 °C which was held for 1 min and was then raised with a ramp of 13 °C/min until 180 °C was reached, followed by a second ramp of 3 °C/min to 250 °C and a third ramp of 20 °C/min to reach the final temperature of 300 °C which was held for 5 min (total run time 41.06 min).

Quantification of 11D5-EE in the sample was performed by means of an external 5-point calibration (c = 0.1–2 μg/mL 11D5-EE). Samples were prepared by dissolving ~10 mg crude DHA-EE oil in 1 mL *n*-hexane. Internal standard (14:0-EE, c = 2 μg/mL) was added to all samples. Peak areas of the extracted base peak (*m*/*z* 179) of 11D5-EE were corrected by the peak area of the extracted ion (*m*/*z* 88) of the internal standard [19].

2.4. Countercurrent chromatography

CCC separations were performed with a Quickprep MK8 system (AECS Downend, UK) equipped with four coils (2.1 mm i.d. stainless steel tubing), located in two opposing bobbins with a β-value ranging from 0.65 to 0.9 [21]. By means of PTFE tubing, the head and tail end of each coil were connected to the front panel of the instrument, so that each coil could be used solely or interconnected to other coils. The measured coil volumes were 116 mL (coil 1), 122 mL (coil 2), 114 mL (coil 3) and 119 mL (coil 4) resulting in a total coil volume of 471 mL [21]. The rotor speed was set to the maximum value (870 rpm) and the temperature during separations was held at 22 °C by external cooling. Both, the stationary and the mobile phase were pumped by means of a ternary beta 50 pump and the effluent of the coils was directed through a Flash 10 diode array detector equipped with a preparative flow-cell (both from Ecom, Praha, Czech Republic). Fractions were collected by means of a Gilson 203 B fraction collector (Middleton, WI, USA).

In the multiple injection mode (Section 3.4) and the repeated multiple injection mode (Section 3.5), a Spider 06 mixing unit (Ecom, Praha, Czech Republic), three T-pieces and four low pressure sample injection valves (all from Sigma Aldrich, Taufkirchen, Germany) were connected by means of PTFE tubing (0.75 mm internal diameter).

The solvent system *n*-hexane – acetonitrile(ACN) (1:1, v/v) was prepared by pouring both solvents into a 2.5 L shake flask and allowing the phases to settle for ~30 min. Then, the phases were separated and degassed via ultrasonication. The CCC system used for the specific separation (coil 1 (116 mL) for normal CCC mode, coil 1–4 (471 mL) for increased throughput CCC and all separations in multiple injection mode, or coil 2 + 4 (241 mL) for the repeated multiple injection mode) was filled with lower (stationary) phase (actual composition 99.5% ACN/0.5% *n*-hexane [22]) (Table 1). Once the selected coil system was completely filled, rotation was started, and the upper (mobile) phase (actual composition 98.8% *n*-hexane/1.2% ACN [22]) was pumped at 1 mL/min (normal CCC mode, multiple injection mode and repeated multiple injection mode), 4 mL/min (increased throughput CCC) or 0.5 mL/min (multiple injection mode with reduced flow rate) into the system (Table 1). The effluent was collected in a graduated cylinder and the volume of the lower (stationary) phase was measured when two distinct phases were visible and equilibrium in the coil was reached to calculate the stationary phase retention (S_f value). When the system was equilibrated the sample was either injected at the beginning of the coil (1 g sample in 10 mL upper phase in normal CCC mode or 4 g in 40 mL for the increased throughput CCC) or at 4 (multiple injection mode) or 2 (repeated multiple injection mode) different points throughout the CCC system (all 1 g sample in 10 mL upper phase) (Table 1). In all modes, injection was done by means of a 10 mL sample loop except the increased throughput CCC where sample was injected by means of a 40 mL sample loop (Table 1). Fractions were collected manually according to the peaks in the UV/Vis chromatogram (λ = 210 nm) or 38 fractions of 2 mL were collected from 10 to 86 min (10–86 mL) in the normal CCC mode.

2.5. Determination of K-values

A small volume of solvent system (~10 mL) was prepared by mixing the solvents in a 20 mL tube and separating them after equilibration. About 100 μL of a standard solution containing DHA-EE and 11D5-EE (c = 400 μg/mL) was placed in an 8 mL tube and solvents were removed by a gentle stream of nitrogen at 40 °C. The residue was taken up in 1 mL of upper and lower phase each. The

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