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On-line hyphenation of centrifugal partition chromatography and high pressure liquid chromatography for the fractionation of flavonoids from *Hippophaë rhamnoides* L. berries

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ABSTRACT

Centrifugal Partition Chromatography (CPC), a liquid–liquid preparative chromatography using two immiscible solvent systems, benefits from numerous advantages for the separation or purification of synthetic or natural products. This study presents the on-line hyphenation of CPC-Evaporative Light Scattering Detector (CPC-ELSD) with High Performance Liquid Chromatography-UV (HPLC-UV) for the fractionation of flavonols from a solvent-free microwave extract of sea buckthorn (*Hippophaë rhamnoides* L., Elaeagnaceae) berries. An Arizona G system was used for the fractionation of flavonoids by CPC and a fused core Halo C18 column allowed the on-line analyses of collected fractions by HPLC. The on-line CPC/HPLC procedure allowed the simultaneous fractionation step at preparative scale combined with the HPLC analyses which provide direct fingerprint of collected fractions. Thus the crude extract was simplified and immediate information on the composition of fractions could be obtained. Furthermore, this methodology reduced the time of post-fractionation steps and facilitated identification of main molecules by Mass Spectrometry (MS). Rutin, isorhamnetin-3-O-rutinoside, isorhamnetin-3-O-glucoside, quercetin-3-O-glucoside, isorhamnetin-rhamnoside, quercetin and isorhamnetin were identified. CPC-ELSD/HPLC-UV could be considered as a high-throughput technique for the guided fractionation of bioactive natural products from complex crude extracts.

1. Introduction

In recent decades, Counter Current Chromatography (CCC), a liquid–liquid chromatography technique, has been increasingly employed for the separation of natural products from plant extracts [1–3]. Its main advantages are a wide injection capacity and the absence of solid support, thus eliminating irreversible adsorption of the sample, which can be fully recovered from the column. Modern CCC apparatus can be divided into two categories, the hydrodynamic equilibrium system and the hydrostatic one. The hydrodynamic system, known as High Speed CCC (HSCCC), uses column composed of one or more coils of Teflon® tubes which rotate about their own axis and also around the centrifuge axis in planetary motion [4]. The use of the hydrostatic system is specific to Centrifugal Partition Chromatography (CPC), in which column is characterized by small elution chambers interconnected by capillary tubing rotating on only one axis [5].

The main use of CCC is the purification of few compounds from natural mixture. However the focus on one or two compounds of interest often leads to the coelution of other compounds. CCC could also be used to fractionate and simplify the whole crude extract; in this case, each fraction contains some of the initial crude extract compounds. Even if collection is followed and monitored by detectors, the composition of the different fractions collected must subsequently be evaluated by a standard chromatography technique such as High Performance Liquid Chromatography (HPLC) or Thin Layer Chromatography (TLC) combined with detectors. In off-line procedure, the fractions collected from CCC are later subjected to HPLC analyses, inducing multiple manual steps such as concentration and/or solvent change resulting in lengthy procedures with a high solvent and material consumption. The two step procedure CCC fractionation and HPLC analysis is a good first way to obtain a better separation of compounds, to avoid coelution and matrix effect and thus facilitate their further identification by Mass Spectrometry (MS).

In recent years, different strategies have been adapted from CCC apparatus in order to resolve complex plant matrices. For example HSCCC and CPC have been hyphenated to MS using an electrospray ionisation (ESI) source [6,7] to obtain direct identification of purified compounds. One of the many developments by Ito et al. [8] has led to the coupling of two HSCCC. Multidimensional CCC (MDCCC) has been applied to the separation of aglycone flavones from *Hip-*

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pophaë rhamnoides and *Gingko biloba* extracts. A bi-dimensional CCC/HPLC-UV was also developed to estimate the hydrophobicity of a microbial extract [9]. Lastly, HSCCC has been monitored on-line with an HPLC-diode array detector (DAD) for the purity control of hyperoside [10] and xanthones [11] isolated from partially purified *Hypericum perforatum* and *Anemarrhena asphodeloides* extracts respectively.

The aim of this study was to develop a separation procedure by hyphenation of CPC and HPLC in order to get an HPLC guided fractionation. The CPC effluent was on-line monitored by HPLC while the fraction collection was carried out in the same time. We demonstrated that it was possible and easy to hyphenate CPC and HPLC to guide fractionation of crude extract at preparative scale. This procedure was performed to separate and on-line guide the fractionation of flavonoids from a sea buckthorn (*H. rhamnoides* L., Elaeagnaceae) berries' extract. On-line analyses of fractionation step provided an instantaneous fingerprint of the CPC effluent and reduced the post fractionation time, as no sample preparation was required before HPLC injection.

2. Materials and methods

2.1. Chemicals

Ultra pure water (H_2O) was first distilled with a Millipore Elix UV system (Saint-Quentin-en-Yvelines, France) and then purified (resistance < 18 MΩ) with an Elgastat UHQ II system (Elga, Antony, France). HPLC grade methanol (MeOH), acetonitrile (ACN), ethyl acetate (EtOAc) and heptane (Hept) were purchased from SDS Carlo Erba (Val de Reuil, France). Acetic acid (CH_3COOH), quercetin-3-O-glucoside and quercetin were supplied by Sigma–Aldrich (Saint-Quentin-Fallavier, France). Rutin, isorhamnetin, isorhamnetin-3-O-glucoside and isorhamnetin-3-O-rutinoside were provided by Extrasynthese (Genay, France).

2.2. Plant material and extraction process

H. rhamnoides L. berries were collected between August and September 2008 from a natural site in Moldavia (Romania). The fresh berries were kept at −20 °C, and before operation they were subsequently defrozen and all their wood parts removed.

Extraction was done by pressurized solvent-free microwave extraction (PSFME) [12], a technique based on the heating of water present inside plant cells under microwave irradiation. As cell walls cannot support the increase in pressure inside the plant cell, the release of a large number of compounds outside the matrix occurs, leading to a complex extract. PSFME was performed in a Milestone MicroSYNTH microwave oven (Sorisole, Italy) equipped with a 50 mL reactor into which about 4 g of *H. rhamnoides* berries was introduced. The fruits were subjected to 5 extraction cycles for 50 s each and to an irradiation power of 1000 W. Between each cycle a cool down step in ice was included and two extracts were pooled for fractionation.

2.3. Equipment

The CPC instrument used during experiments was a Semi-preparative FCPC® from Kromaton (Angers, France), equipped with a 200 mL hydrostatic column and coupled with a SEDEX 45 Evaporative Light Scattering Detector (ELSD) purchased from SEDERE (Alfortville, France). To monitor the CPC effluent, it was divided using a variable flow splitter (VFS) from Rheodyne (Rohnert Park, CA, USA). The VFS principle consists in using an active switching device that transfers a small volume from the CPC effluent into a separate and independent auxiliary stream directed to the ELSD detector. This auxiliary stream flow was supplied by a one-way LC-10 AS pump (Shimadzu, Japan) which delivered a 50:50 (MeOH:H_2O) solution at a flow rate of 0.3 mL min^{-1}.

All HPLC experiments were conducted with an Agilent HP 1100 apparatus (Waldbronn, Germany) coupled to a Kontron Ultra-Violet (UV) detector (Zurich, Switzerland) and piloted by EZchrome Elite workstation software.

Both CPC and HPLC were coupled with a six-port switching valve (Valco Instruments, Houston, USA) equipped with a 20 μL injection loop.

A Quattro Ultima Micromass triple quadrupole equipped with a Z-spray dual orthogonal electrospray source from Waters (Saint Quentin, France) was used for the identification of compounds.

2.4. CPC procedure

CPC is a separation technique that uses two immiscible solvent phases, one of which remains stationary while the other is mobile. The CPC column contains a stacked circular disk made of a succession of small cells linked in cascade by capillary ducts. The liquid phase chosen as the stationary one is first introduced into the CPC column and kept stationary by centrifugal force generated by the rotation of the system. The mobile phase is then pumped through the stationary phase until equilibrium is attained. Selection of the two-phase solvent system was made by calculating the partition coefficient $K(C_{Upper}/C_{Lower})$ of the compounds of interest, defined as the concentration of analyte in the upper phase (C_{Upper}) divided by the concentration of solute in the lower phase (C_{lower}). Five Arizona systems were tested (C, F, G, H and K) [13] and the best one to obtain an overall separation of analytes was the G Arizona system composed of Hept/EtOAc/MeOH/H_2O (1/4/1/4) [13]. The stationary phase was the more hydrophilic one (aqueous lower phase) and the mobile phase the more hydrophobic one (organic upper phase). Approximately 1.5 g of extract diluted in an 8 mL mobile phase/stationary phase mixture (50:50) was injected into the column via a six-port medium-pressure injection valve (Upchurch Scientific, Oak Harbor, WA, United States) equipped with a 10 mL loop.

The separation parameters were optimized to obtain the best compromise between retention of the stationary phase, fractionation time and backpressure. Separation was performed at 1300 rpm. The mobile phase flow was set at 3 mL min^{-1} and elution was carried out for 100 min in ascending mode (mobile organic phase pumped through the column from tail to head). Afterward an extrusion step was achieved, the fresh aqueous stationary phase was pumped in ascending mode to extrude first the organic mobile phase and then the aqueous stationary phase contained in the column, until all the compounds had been extruded from the column [9]. During extrusion, the flow rate and rotation were respectively decreased to 2 mL min^{-1} and 500 rpm.

2.5. HPLC separation

To obtain a rapid HPLC analysis, we evaluated two columns dedicated to fast analysis: the monolithic Chromolith Performance RP-18e (100 × 4.6 mm ID, 2 μm) provided by Merck (Darmstadt, Germany) and the fused core HALO C18 column (50 × 4.6 mm ID, 2.7 μm) supplied by Interchim (Montluçon, France), which can be used both at high pressures and at high flow rates. Monolithic columns are characterized by a bimodal pore distribution composed of macropores and mesopores [14], while fused core columns are filled with 2.7 μm particles, composed of a 1.7 μm solid core surrounded by a 0.5 μm porous silica layer [15]. The mobile phase was a mixture of water (A) and acetonitrile or methanol (B) used under a linear gradient from 95% of A at 0 min to 55% of A at 10 min. A and B were both 1% acidified with CH_3COOH. The flow

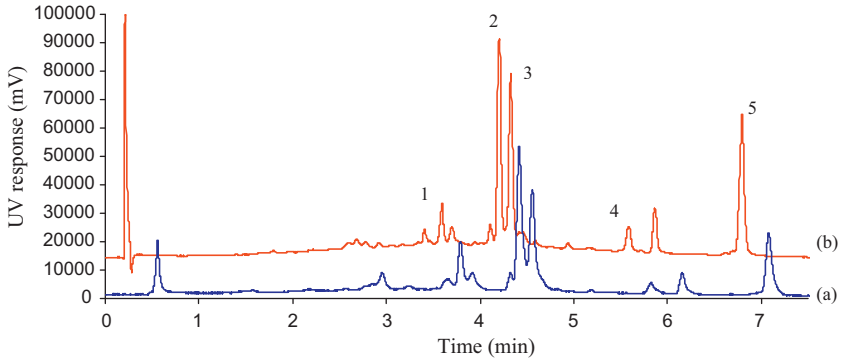

Fig. 1. HPLC chromatograms of the crude extract obtained at 366 nm with the (a) Chromolith Performance RP-18e column (100 × 4.6 mm ID, 2 μm) at 3 mL min^{-1} and with the (b). Fused core HALO C18 column (50 × 4.6 mm ID, 2.7 μm) at 2 mL min^{-1}. Mobile phase H_2O/ACN both acidified with 1% CH_3COOH, 40 °C. Linear gradient from 95% to 55% of H_2O. Rutin (1), isorhamnetin-3-O-rutinoside (2), isorhamnetin-3-O-glucoside (3), quercetin (4) and isorhamnetin (5).

rate was tested from 1 to 3 mL min^{-1} depending on the column. Column temperature was controlled by an Interchim Crococil oven (Montluçon, France). UV detection was recorded at 366 nm, the characteristic wavelength of flavonols. Off-line HPLC optimisation was done by injecting crude extract diluted in a MeOH:H_2O mixture (50:50). The injection volume was 20 μL except when its influence was studied.

2.6. Liquid Chromatography–Mass Spectrometry (LC–MS) identification

LC analyses were conducted on an Altima reversed-phase C18 column (150 × 4.6 mm ID, 5 μm) purchased from Alltech (Deerfield, USA) at room temperature with a flow rate of 1 mL min^{-1} and a 20 μL injection loop. The mobile phase was water (solvent A) and methanol (solvent B), both acidified with 1% of CH_3COOH using a gradient program of 5% B for 5 min, 5–20% B for 15 min, 20% B for 5 min, 20–50% B for 10 min, and 50% B for 10 min.

The electrospray source was used in negative ionisation mode. The 1 mL min^{-1} flow rate from the LC device was split with a microvalve T-splitter (Upchurch Scientific, Oak Harbor, USA) to a flow rate of about 0.3 mL min^{-1} directed to the MS system. Optimal conditions of flavonol fragmentation were found by direct infusion of specific standards (quercetin, quercetin-3-glucoside, rutin). The simple MS measurements were therefore made using a −4.5 kV capillary voltage, a −35 V cone voltage, a source temperature of 400 °C, a desolvation temperature of 96 °C, a flow of 80 L h^{-1} for the cone and of 55 L h^{-1} for the desolvation gas (nitrogen). From each CPC fraction a first LC–MS full scan, from *m/z* 50 to *m/z* 1000 uma, was done; thus the most intense ions detected at different retention times were selected as target ions to be fragmented using collision energy set at 25 eV with argon as collision gas.

3. Results and discussion

3.1. HPLC separation conditions

In order to obtain the quickest second dimension using a conventional HPLC apparatus, different well known factors which can influence the separation were investigated on Chromolith Performance RP-18e and the fused core HALO C18 column: the nature of organic solvents, the effect of the mobile phase flow rate and the effect of column temperature. Due to the wide polarity of molecules to be analysed, a linear gradient was required to obtain good separation in HPLC. Acetonitrile was chosen as organic solvent because it induces a lower backpressure and a shorter analysis time than methanol for both columns. Different flow rate values were tested from 1 to 3 mL min^{-1}. Optimal conditions were found with a flow rate of 3 mL min^{-1} and 2 mL min^{-1} respectively for the monolithic column and the fused core column. However, operating at a flow rate of 2 mL min^{-1} was preferable to 3 mL min^{-1} because less solvent was consumed and sensitivity was increased due to a lower sample dilution in the mobile phase.

Results obtained with the two columns showed that the use of a higher temperature than ambient temperature decreased the retention time and the backpressure (data not shown). For both columns, temperature was set at 40 °C. Under optimal conditions (Fig. 1) an analysis time of 6.7 min (H_2O/ACN, 2 mL min^{-1}, 40 °C) was observed on the fused core column and of 7.1 min (H_2O/CAN, 3 mL min^{-1}, 40 °C) on the monolithic column. Re-equilibration time after gradient elution was also considered, giving a total run time shorter for the fused core (7.3 min) than for the monolithic column (8.2 min). The fused core column was consequently chosen for the further hyphenation. A total run time lower than 10 min was suitable for an on-line CPC/HPLC coupling because the preparative scale of CPC leads generally to a total elution time of CPC peaks about 10–20 min. Each CPC peak could thus be analysed at least once or several times by HPLC.

3.2. Effects of injection solvent, volume injected and amount injected on HPLC separation

The biphasic system and the preparative scale of CPC could lead to some drawbacks, when building an on-line chromatographic CPC/HPLC system, such as miscibility of the two mobile phases used in each separation device (the mobile phase of the CPC was the injection solvent of the HPLC), the maximum volume which could be injected and the effect of the amount injected into the HPLC column. These parameters were investigated in order to avoid band widening and peak deformation. The Arizona G system Hept/EtOAc/MeOH/H_2O (1/4/1/4) found as the best among five Arizona systems, was used in ascending mode for the separation of sea buckthorn flavonols by CPC. According to the results reported by Berthod et al. [13], the lower phase of this biphasic system is mostly composed of H_2O and MeOH, and the upper phase of EtOAC and Hept. In the worst case, which was our, the organic phase was the mobile phase of the CPC fractionation and became the injection solvent of HPLC. Thus, injection of the upper organic phase into the HPLC mobile phase (H_2O:ACN, 95/5, v:v both acidified with 1% CH_3COOH) could generate a biphasic injection solvent and/or emulsion which could further degrade LC separation. Furthermore, mixing mobile phases with different viscosities could induce a flow instability known as viscous fingering [16], producing distorted peaks or multiple peaks for a single analyte.

In order to study the effect of the injection solvent nature, a five flavonol standard mixture (rutin (1), isorhamnetin-3-O-rutinoside

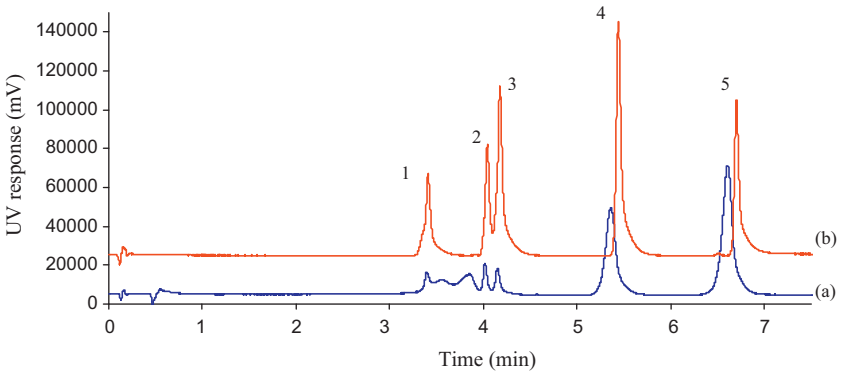

Fig. 2. HPLC chromatograms obtained at 366 nm with the fused core HALO C18 column (50 × 4.6 mm ID, 2.7 μm) at 2 mL min^{-1} by injection of 20 μL standard mixture either (a) in the upper organic phase or (b) in the lower aqueous phase. Mobile phase H_2O/ACN both acidified with 1% CH_3COOH, 40 °C. Linear gradient from 95% to 55% of H_2O. Rutin (1), isorhamnetin-3-O-rutinoside (2), isorhamnetin-3-O-glucoside (3), quercetin (4) and isorhamnetin (5).

(2), isorhamnetin-3-O-glucoside (3), quercetin (4) and isorhamnetin (5)) was selected as they had been previously identified in sea buckthorn berries [17]. This mixture was diluted either in the lower aqueous phase or in the upper organic phase and then injected onto the HPLC device (20 μL, 0.4 mg mL^{-1}). Fig. 2 illustrates the HPLC results obtained on fused core column. The injection of solutes diluted in the upper organic phase (Fig. 2a) led to a deformation in the chromatographic peaks corresponding to glycoside flavonols (1, 2 and 3) and the occurrence of viscous fingering. In the case of the more hydrophobic aglycone molecules (4 and 5), the effect of injection in organic solvent was limited to band widening. Thus the injection of quite non-polar molecules onto a reversed-phase HPLC system (H_2O:ACN both acidified with 1% CH_3COOH) would be possible even if they were diluted in a Hept/EtOAC solvent. When the standard mixture was injected in the lower aqueous phase (Fig. 2b) rather than in the upper one, a better efficiency and separation were observed.

The injection of 50–100 μL of standard mixture diluted in the aqueous lower phase caused shouldering and broadening of the chromatographic peaks corresponding to flavonol glycosides (data not shown), but injection of a 20 μL volume did not induce any viscous fingering. Thus 20 μL was therefore defined as the appropriate transferable volume from the CPC outlet to the HPLC column.

CPC is a semi-preparative chromatographic technique compatible with sample loads on the order of grams, whereas HPLC is an analytical technique (injection of μg loads). To avoid HPLC column surcharge, the maximum amount that could be transferred between the two chromatographic systems was assessed before hyphenation. The injection of 1.5 g crude extract solution into CPC column might lead to a highly concentrated effluent at the CPC outlet. To evaluate if it was possible to inject highly concentrated effluent into HPLC column, crude extract diluted in a MeOH:H_2O mixture (50:50) was injected in the HPLC system at different concentrations (1, 5, 10, 50 and 100 mg mL^{-1}). Fig. 3 shows that injection of 50 and 100 mg mL^{-1} of crude extract did not affect HPLC separation. The same results were observed with the injection of 1, 5, and 10 mg mL^{-1}.

The injection volume appeared as the main limiting factor and consequently CPC effluent could be directly injected in the HPLC flow whatever is composition (upper or lower phase) and is concentration (tested up to 100 mg mL^{-1}) if the injection volume does not exceed 20 μL.

3.3. Development of the on-line system

Prior to developing the direct coupling of CPC and HPLC it was necessary to consider possible incompatibility between these two techniques in terms of backpressure and flow rate values. CPC is a low-pressure chromatography operating at a pressure that cannot exceed 60 bars in our case, whereas HPLC is a high-pressure chromatography using classical apparatus compatible with a pressure of 400 bars. In our optimal conditions, the CPC pressure equilibrium was 38 bars, and the HPLC pressure equilibrium was 184 bars with the fused core column. The two chromatographic methods used also differ in their current operating flow rate: FCPC® can be employed up to 15 mL min^{-1}, whereas HPLC is generally used at 1 mL min^{-1}. Constructing a multidimensional system with these constraints could therefore be expected to lead to problems of

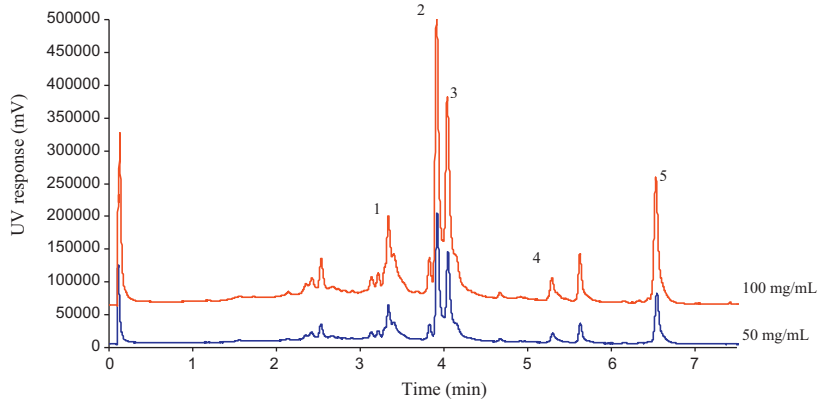

Fig. 3. HPLC chromatograms of the crude extract at different concentrations (50 and 100 mg mL^{-1}) obtained at 366 nm with the fused core HALO C18 column (50 × 4.6 mm ID, 2.7 μm) at 2 mL min^{-1}. Mobile phase H_2O/ACN both acidified with 1% CH_3COOH, 40 °C. Linear gradient from 95% to 55% of H_2O. Rutin (1), isorhamnetin-3-O-rutinoside (2), isorhamnetin-3-O-glucoside (3), quercetin (4) and isorhamnetin (5).

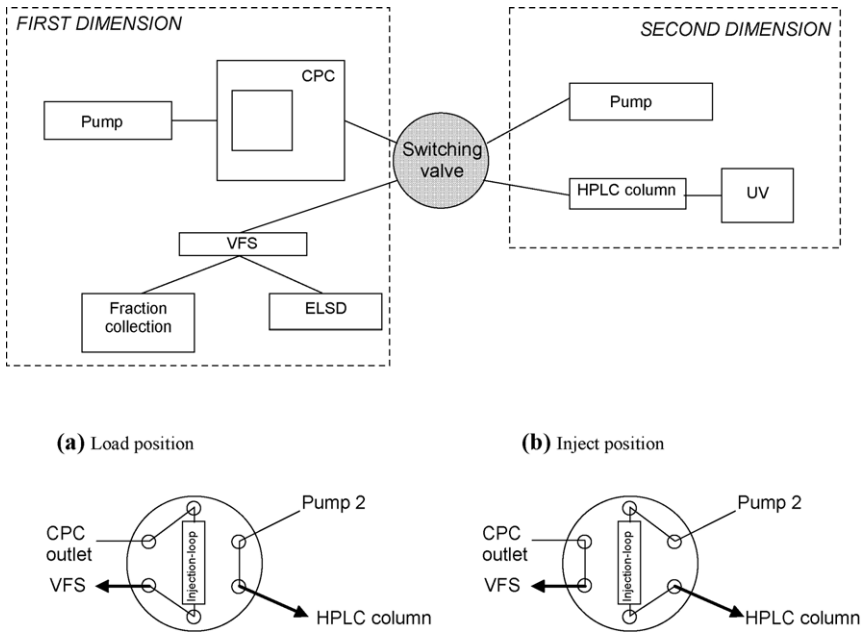

Fig. 4. Schematic diagram of the on-line CPC/HPLC system. FCPC® 200 mL (1300 rpm, 3 mL min^{-1}, 38 bars, ascending mode then extrusion) coupled to a fused core HALO C18 column (50 × 4.6 mm ID, 2.7 μm, H_2O/CAN both acidified with 1% CH_3COOH, 2 mL min^{-1}, 40 °C) using a manual six-port switching valve equipped with a 20 μL injection loop. (VFS) Variable flow splitter. (a) Six-port switching valve configuration in position "load". (b) Six-port switching valve configuration in position "inject".

watertightness and leakage. These problems were resolved by the construction of the on-line CPC/HPLC system illustrated in Fig. 4. Switching between the CPC and the HPLC was achieved using a manual Valco six-port valve which enabled physical separation between the two chromatographic systems. When the valve was in 'load' position the CPC outlet was sent through the injection loop to the VFS system (Fig. 4a). Effluent was split in two: one part was passed through ELSD detection and the other through fraction collection. When the valve was in 'inject' position, the effluent from CPC present in the injection loop was sent to the HPLC (Fig. 4b), during that time CPC continued to operate with the CPC outlet sent to VFS system.

3.4. HPLC guided CPC fractionation

The CPC fractionation was performed from a 1.5 g crude extract of *H. rhamnoides* berries dissolved in a 50:50 (mobile phase:stationary phase) mixture. During the 180 min fractionation time, 24 cuts (C_x) from the CPC to the HPLC were done, i.e. one cut every 7.5 min. The CPC-ELSD chromatogram (Fig. 5a) shows two major peaks corresponding to apolar and polar compounds recorded respectively before and after extrusion. A fraction collection based only on ELSD monitoring would have led to two major fractions (the first one between 20 min and 50 min and a second one between 140 min and 170 min). Using HPLC-UV in on-line coupling allowed guiding of fractionation step as CPC effluent could be instantaneously analysed. This can be seen in Fig. 5b which shows the relevant HPLC-UV fingerprints obtained from the CPC effluent. The cuts C_3 and C_4 clearly show that different compounds co-eluted under one CPC peak. The hyphenated method allowed to collect two fractions for this CPC peak. The first one from cut C_3 that contained compounds 4 and 5 (solute 5 more concentrated in the crude extract was the majority solute), and a second one from C_4 with solute 6 and a few amount of solute 5. Furthermore, on-line HPLC analyses (C_6–C_{19},) of the part without ELSD response (between 50 and 140 min), which seems without interest, clearly showed that minor solutes could be concentrated, separated and collected in fractions. Indeed compound 7 could be isolated from C_6 and compound 3 from C_{16} and C_{17} and another fraction gathered solutes 8 and 3. However, in cuts between C_6 and C_{16} no molecule had been detected. HPLC fingerprints (C_{20} and C_{21}) indicate that compounds 1 and 2 were coeluted under the CPC peak corresponding to the extrusion step. As C_{20} shows, compound 2 could be collected as

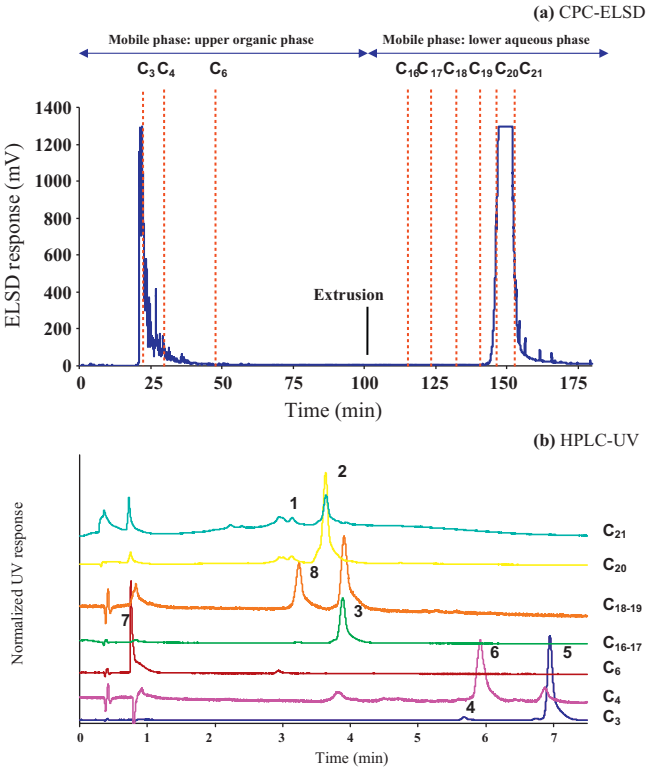

Fig. 5. Chromatograms obtained with CPC-ELSD (a) and on-line HPLC-UV at 366 nm (b) on a fused core HALO C18 column (50 × 4.6 mm ID, 2.7 μm) at 2 mL min^{-1}. Mobile phase H_2O/ACN both acidified with 1% CH_3COOH, 40 °C. Linear gradient from 95% to 55% of H_2O C_x corresponds to relevant cuts done from CPC effluent to HPLC. Rutin (1), isorhamnetin-3-O-rutinoside (2), isorhamnetin-3-O-glucoside (3), quercetin (4) and isorhamnetin (5), isorhamnetin-rhamnoside (6), unidentified compounds (7), quercetin-3-O-glucoside (8).

Table 1
Retention time (tr) and characteristic ions of flavonols from *Hippophaë rhanmoides* berries extract detected by LC–MS.

Cut	Peak	tr (min)	[M−H]− (*m/z*)	MS–MS (*m/z*)	Name
3	5	6.9	315	301, 151	Isorhamnetin
	4	5.4	301	151, 121, 179	Quercetin
4	6	5.9	461	315	Isorhamnetin-rhamnoside
6	7	0.8	–	–	Unidentified
16–17	3	3.9	477	314	Isorhamnetin-3-glucoside
18–19	3	3.9	477	314	Isorhamnetin-3-glucoside
	8	3.2	463	300	Quercetin-3-O-glucoside
20–21	1	3.1	609	300	Quercetin-3-rutinoside
	2	3.6	623	315	Isorhamnetin-3-rutinoside

majority solute at the beginning of the CPC peak, but at the end compounds 2 and 1 were present in similar proportion (C_{21}) mm.

HPLC chromatograms indicate that no peak deformation occurred during elution–extrusion hyphenation, thus the system used enabled the connection of CPC and HPLC either in orthogonal (reversed and normal phases) or in non-orthogonal (reversed or normal phases) separation mechanisms. Previous multidimensional developments on CCC apparatus [8,10,11] were not realized in elution–extrusion mode leading to a longer separation time. Moreover all the systems were coupled using reversed mode aqueous mobile phase for CPC and HPLC separations. On-line CPC/HPLC eliminates the sample multistep treatments required in off-line (e.g. concentration, solvent change, vial transfer) and consequently decrease the time of work.

3.5. Mass Spectrometry identification

The collected fractions were further submitted to LC/MS analyses to characterize flavonol extracted and separated from *H. rhamnoides* berries. MS identification of flavonol was facilitated, as no coelution, matrix effect and signal extinction were observed. Identification of flavonols was based on their MS/MS spectra and their retention time in comparison with flavonol standards tested in the same conditions (Table 1). Isorhamnetin (5) and quercetin (4) were found to be abundant not only in C_3, isorhamnetin-rhamnoside (6) in C_4, isorhamnetin-3-O-glucoside (3) in $C_{16–17}$, but also in $C_{18–19}$ with quercetin-3-O-glucoside (8), whereas di-glycoside flavonols such as rutin (1) and isorhamnetin-3-O-rutinoside (2) were detected in $C_{20–21}$. One unknown compound (7) was also found in C_6 but remains unidentified for now. The previous CCC separation of *H. rhamnoides* berries has led to the identification of isorhamnetin, quercetin [8], and of glycoside flavonols quercetin-3-O-glucoside, isorhamnetin-3-O-glucoside and isorhamnetin-3-O-rutinoside [6]. Using the HPLC guided CPC fractionation, we reported the presence of these molecules but we also detected rutin, isorhamnetin-rhamnoside and compound 7 not identified for now.

4. Conclusion

This present work described for the first time how to hyphenate CPC with HPLC using a fast HPLC run for the fractionation of the main flavonols from *H. rhamnoides* berries. The innovative on-line separation approach (CPC-ELSD/HPLC-UV) allows to guide fractionation step at preparative scale with direct fingerprint of collected fractions. The procedure led to the collection of the main flavonols of sea buckthorn berries in different fractions. This methodology eliminated the concentration step, reduced the post-fractionation time by direct on-line analyses of the collected fractions. Due to its high versatility, CPC can be used easily either as a normal phase chromatography (polar stationary phase) or as a reversed-phase chromatography (apolar stationary phase) allowing CPC and HPLC coupling in orthogonal or in non-orthogonal separation mechanisms. Thus, CPC offered a wide range of possibilities for the construction of a hyphenated system with HPLC, and constituted a high-throughput technique for the analysis of natural products from complex crude extracts.

In the future, the hyphenation of CPC/HPLC with mass spectrometry should be an important tool in the studies of complex natural extracts in order to obtain direct structural information on separated compounds. This multidimensional technique can be considered as a powerful technique which can be used on a daily basis as the technique of choice to fractionate complex samples. One of the trends which will enable CCC to overcome its present drawbacks could therefore be its automatic hyphenation with LC systems, promoting the easy and widespread use of the methodology.

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