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Intensified extraction of ionized natural products by ion pair centrifugal partition extraction

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ABSTRACT

The potential of centrifugal partition extraction (CPE) combined with the ion-pair (IP) extraction mode to simultaneously extract and purify natural ionized saponins from licorice is presented in this work. The design of the instrument, a new laboratory-scale Fast Centrifugal Partition Extractor (FCPE300®), has evolved from centrifugal partition chromatography (CPC) columns, but with less cells of larger volume. Some hydrodynamic characteristics of the FCPE300® were highlighted by investigating the retention of the stationary phase under different flow rate conditions and for different biphasic solvent systems. A method based on the ion-pair extraction mode was developed to extract glycyrrhizin (GL), a biologically active ionic saponin naturally present in licorice (*Glycyrrhiza glabra* L., Fabaceae) roots. The extraction of GL was performed at a flow rate of 20 mL/min in the descending mode by using the biphasic solvent system ethyl acetate/*n*-butanol/water in the proportions 3/2/5 (v/v/v). Trioctylmethylammonium with chloride as a counter-ion (Al336®) was used as the anion extractant in the organic stationary phase and iodide, with potassium as counter-ion, was used as the displacer in the aqueous mobile phase. From 20 g of a crude extract of licorice roots, 2.2 g of GL were recovered after 70 min, for a total process duration of 90 min. The combination of the centrifugal partition extractor with the ion-pair extraction mode (IP-CPE) offers promising perspectives for industrial applications in the field of natural product isolation or for the fractionation of natural complex mixtures.

1. Introduction

Centrifugal partition chromatography (CPC) refers to a particular type of support-free liquid–liquid chromatographic techniques where solutes are separated on the basis of their partition coefficients between two immiscible solvent systems [1,2]. The particularity of CPC systems arises from their column which contains a series of interconnecting cells mounted circumferentially on stacked disks in rotation around a single axis [1]. One liquid is maintained inside the column as the stationary phase by a constant centrifugal force field while the other phase is pumped through it. This technique is very attractive in terms of selectivity, sample loading capacity and scaling-up ability [3,4]. CPC has been widely used for the extraction and purification of natural products [5–8]. Many processes and equipments are currently being developed to transpose these techniques at the industrial scale [2,9,10]. The main challenge is to reduce the process duration and consequently to increase flow rates and mass sample loading.

In this study, we used an original device, the Fast Centrifugal Partition Extractor (FCPE300®) that has recently been designed by Rousselet Robatel Kromaton (Angers, France). This laboratory-scale extractor has evolved from centrifugal partition chromatography (CPC), but its column contains less cells of larger volume (231 twin cells, each twin cell representing a single mixing and settling stage). The aim of this work was to examine the potential of the FCPE300® apparatus for the extraction and purification of natural ionic compounds. For this purpose, a method based on the ion-pair extraction mode was developed on the centrifugal partition extractor (IP-CPE). The inspiration for ion-pair extraction came from a strong ion exchange method previously developed in combination with centrifugal partition chromatography (SIXCPC) for the isolation and purification of glucosinolates from white mustard and broccoli seed aqueous extracts [11] and rosmarinic acid from *Lavandula vera* cell cultures [12]. After investigating the effect of flow rates on the retention of the stationary phase for different biphasic solvent systems, the IP-CPE method was applied to the extraction of glycyrrhizin (GL), an ionized triterpene saponin present in 1–12% of the dry hydro-alcoholic extracts of licorice roots (*Glycyrrhiza glabra* L., Fabaceae) [13,14]. GL comprises two molecules of glucuronic acid linked to the glycyrrhetic acid aglycone moiety and

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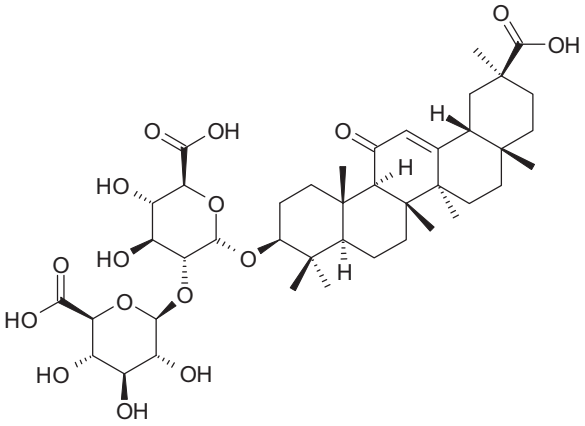

Fig. 1. Glycyrrhizin: (3β,18α)-30-hydroxy-11,30-dioxoolean-12-en-3-yl-2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranosiduronic acid.

occurs naturally in a salt form (calcium, magnesium or potassium) (Fig. 1).

Various other secondary metabolites are present in licorice roots, among which flavanones (liquiritin and glabrol derivatives), isoflavanes (glabridine and derivatives) and chalcones (isoliquiritin, licuraside) [15–17]. Several studies conducted on animal models and in humans have demonstrated the hepatoprotective, anti-inflammatory, anti-allergic, anti-ulcer and antimicrobial activities of GL and licorice root extracts [18,19]. Several extraction and separation methods for GL have been developed, including macro-porous resin separation, microwave-assisted extraction, pressurized hot water extraction, as well as high-speed and multi-stage countercurrent chromatography [20–23]. However, at present, the purification of GL requires several steps and its recovery is generally only about 20–30% [24]. We have applied the IP-CPE method to recover GL from 20 g of a crude extract of licorice roots in only one lab-scale operation. This innovative combination offers promising perspectives for industrial application due to its potential in terms of process duration, mass sample loading, extraction recovery and purity.

2. Experimental

2.1. Reagents

All analytical grade solvents were used: ethyl acetate (EtOAc), *n*-heptane, *n*-butanol (*n*-BuOH), acetonitrile (CH_3CN), methanol (MeOH), ammonia (NH_3) and chloroform ($CHCl_3$) were purchased from Carlo Erba Reactifs SDS (Val de Reuil, France). Aliquat336® (trioctylmethylammonium chloride, Al336) was purchased from Sigma–Aldrich (Saint-Quentin, France) as a mixture of C_8 and C_{10} chains with C_8 predominating. Potassium iodide was obtained from Prolabo (Fontenay, France). The standard molecule of GL (monoammonium salt, purity >98%, M = 839) was purchased from Extrasynthèse (Genay, France).

2.2. Instrumentation

The laboratory scale Fast Centrifugal Partition Extractor FCPE300® (Kromaton Technology, Angers, France) was equipped with a rotor made of 7 circular partition disks containing a total of 231 partition twin cells (Fig. 2). The distance from the center of the rotor to the center of twin cells is 98.8 mm, and the total diameter of the rotor is 250 mm. The volume of the column is about 300 mL (see Section 3.1). The twin cells are connected together by ducts of 0.8 mm internal width. Rotation speed can be adjusted

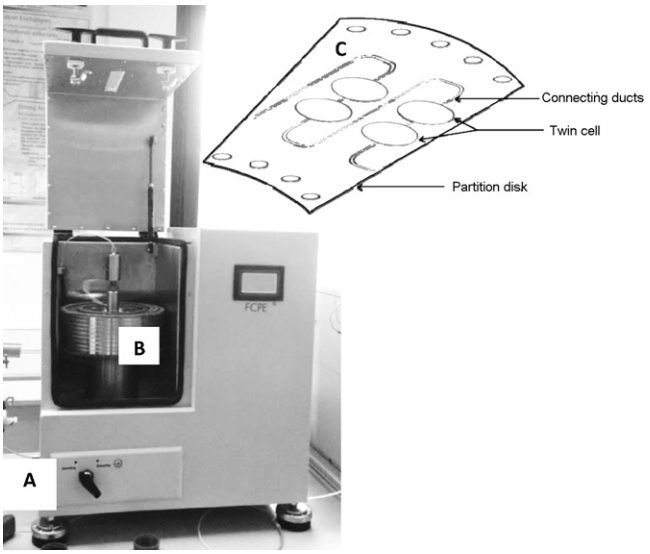

Fig. 2. Kromaton Technology FCPE300®. (A) Laboratory-scale extractor design, (B) partition disks and (C) geometry of the twin partition cells.

from 200 to 2000 rpm, producing a relative centrifugal acceleration in the partition cell up to 437 × *g*. The mobile phase was pumped either in the ascending or in the descending mode with low residual pulsation through a KNAUER Preparative Pump 1800® V7115 (Berlin, Germany). This pump can deliver constant flow up to 1000 mL/min at a maximum pressure of 400 bar. The system was equipped with an Isco500D Syringe Pump of 507 mL cylinder capacity delivering flow rates up to 204 mL/min at pressures up to 262 bars (Teledyne IscoInc., Lincoln, Nebraska, USA). The system was coupled to a UVD 170S detector (Dionex, Sunnivale, CA, USA) equipped with a preparative flow cell (6 μL internal volume, 2 mm path length). Fractions were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). All experiments were conducted at room temperature (20 ± 2 °C).

2.3. Characterization of the Fast Centrifugal Partition Extractor (FCPE300®)

The volume of the FCPE300® column was measured by using a highly stable biphasic solvent system *n*-heptane/water. The column was filled with the aqueous phase saturated with *n*-heptane in the ascending mode at 50 mL/min (column rotation speed: 200 rpm) and subsequently flushed with the organic phase saturated with water in the descending mode. The column volume was measured as the quantity of the aqueous phase evacuated from the column. The experiment was repeated 10 times. Stationary phase retention was evaluated at different flow rates by using the biphasic solvent systems *n*-heptane/water (1/1, v/v), *n*-heptane/MeOH (1/1, v/v), *n*-butanol/water (1/1, v/v), EtOAc/water (1/1, v/v), M*t*BE/water (1/1, v/v), and EtOAc/*n*-butanol/water (3/2/5, v/v/v). Each solvent system was prepared in a separatory funnel. The solvent mixture was vigorously shaken and allowed to settle until phase separation. The column was filled with the organic stationary phase and the rotation speed was adjusted to 1000 rpm. The aqueous mobile phase was then pumped in the descending mode at flow rates ranging from 10 to 300 mL/min in independent experiments without sample injection. For each condition, the percentage of the stationary phase retention was determined by measuring the volume of the stationary phase remaining in the column relative to the total column capacity. All experiments were repeated in the ascending mode and the results were not significantly different.

Table 1
Experimental conditions for EPC process development.

	Experiment	Flow rate (mL/min)	Loaded volume (mL)	Loaded standard (mg), (mmol)	SF (%)	$C_{Al336} = C_{KI}$ (mM); $(n_{Al336} = n_{KI})/n_{GL}$	GL back extraction duration (min)	Total process duration (min)	GL outlet concentration (mM)
Flow rate variation experiments	1	10	20	200 (0.238)	67.21	8.7 (7.4)	7.5	75	3.17
	2	20	20	200 (0.238)	64.9	9.0 (7.4)	3.5	38	3.09
	3	50	20	200 (0.238)	62.43	9.3 (7.4)	1.5	23	3.09
Loaded volume experiments	1′	20	20	131 (0.156)	64.7	3.3 (4.2)	3.3	39	2.34
	2′	20	1000	131 (0.156)	63.9	3.3 (4.2)	2.8	85	2.78
	3′	20	2000	131 (0.156)	63.2	3.3 (4.2)	2.4	136	3.25

C_{Al336}: extractant concentration; C_{KI} = displacer concentration; n_{Al336}/n_{KI} = extractant/displacer molar ratio. See Fig. 5 for the effect of the flow rate on the extraction profile of GL.

2.4. Influence of flow rates on the extraction profile of GL

An ion pair extraction method was developed by using the solvent system EtOAc/*n*-butanol/water (3/2/5, v/v/v). The objective here was to determine the optimum flow rate for a short and efficient extraction of GL. The column was filled with the organic stationary phase containing the extractant Aliquat336® (Al336). This lipophilic quaternary ammonium salt is able to extract anionic compounds in the organic stationary phase while keeping a good retention of the stationary phase, in spite of its surfactant properties [12]. GL standard (200 mg, 0.238 mmol) was dissolved in 19 mL of the fresh aqueous mobile phase and 1 mL of the organic phase. As GL contains three potential ionic sites and Al336 only one ionic site, a large excess of Al336 was added to the stationary phase to ensure the complete extraction of GL. The concentration of Al336 was fixed at about 9 mM (molar ratio $n_{Al336}/n_{GL} \approx 7$). The solution was adjusted to pH 7 and loaded into the column. The mobile phase was pumped in the descending mode at 10, 20 or 50 mL/min in independent experiments (Table 1). The concentration of Al336 was adjusted to the stationary phase retention value obtained for each flow rate tested in order to keep the molar ratio n_{Al336}/n_{GL} constant. After eluting one column volume, potassium iodide (KI) was added to the mobile phase for the back extraction step at a molar ratio $n_{Al336}/n_{KI} = 1$. Iodides were selected as displacer agents due to their higher affinity for the extractant Al336 than GL, resulting in a step-by-step transfer of GL from the stationary to the mobile phase and its progression along the column. The extraction/back extraction process of ionic analytes inside the FCPE300® column is described in Fig. 3. As the analytes are expelled from the stationary phase by the displacer, a series of successive bands is formed and moves along the column at the velocity of the displacer front. This series of bands is called the isotachic train. Each rectangular band refers to analytes of high concentration and high purity. The UV spectral profiles monitored at 252 nm were compared between each flow rate condition.

2.5. Concentration profile of GL inside the FCPE300® column before the back extraction process

Three concentrations of Al336 (3.3 mM, 10 mM or 30 mM) were preliminary tested in order to determine the optimum molar ratio n_{Al336}/n_{GL} which is required to achieve the complete extraction of GL. For that we have examined the condition where all the sites of Al336 are occupied by GL (as reflected by an excess of GL out of the column) and subsequently calculated the molar quantities of Al336 and GL remaining inside the column in this particular situation.

The concentration profile of GL inside the FCPE300® column was then determined by extrusion [25]. After loading 200 mg of GL standard into the system, one column volume of the aqueous mobile phase (without KI) was pumped in the descending mode at 10 mL/min. The organic stationary phase was then extruded by pumping a new column volume of the aqueous mobile phase in

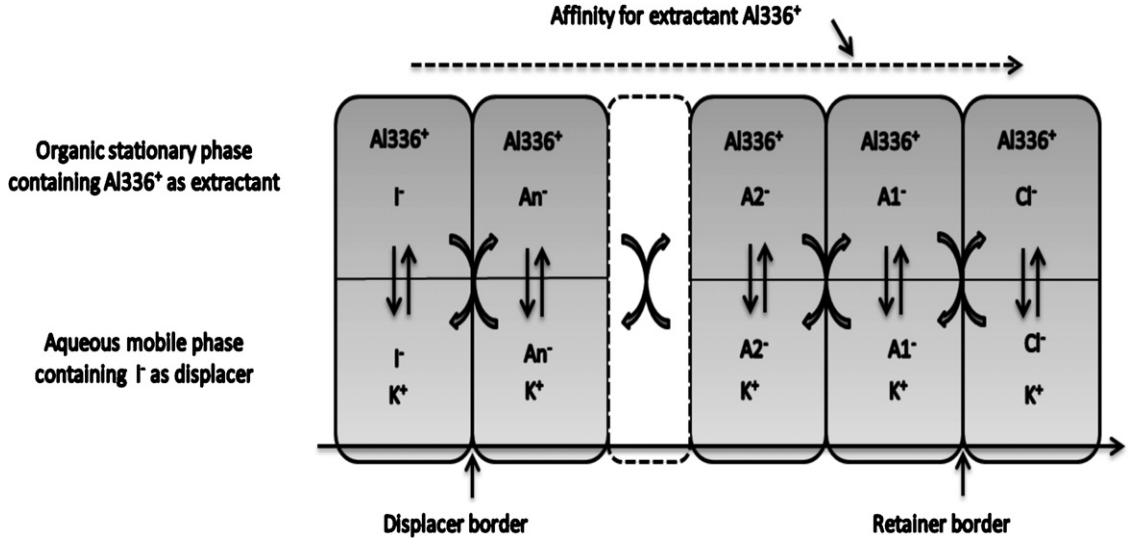

Fig. 3. Progression of the isotachic train during the ion-pair extraction/back extraction process inside the FCPE300® column. Separation of nA^- anions (A_1^-, A_2^-,. . ., A_n^-) using ($Al336^+$) as anion-extractant and iodides (I^-) as displacer (Cl^-: retainer and K^+: displacer counter-ions).

the ascending mode at 10 mL/min. The amount of glycyrrhizin was determined by HPLC in the successive fractions (20 mL each) collected at the column outlet.

2.6. Influence of the inlet sample volume

The influence of the feed volume on the extraction profile and outlet concentration of GL was investigated. The concentrations of Al336 in the stationary phase and of KI in the mobile phase were fixed at 3.3 mM (n_{Al336}/n_{GL} = 4.2). In three independent experiments, GL standard was dissolved in 20 mL, 1000 mL or 2000 mL of the aqueous mobile phase and loaded into the CPE column. Experimental conditions are summarized in Table 1. The displacer KI was added at a molar ratio n_{Al336}/n_{KI} = 1 and pumped at 20 mL/min until all GL was back extracted.

2.7. Preparation of the crude extract of licorice roots

Powdered licorice roots (510 g, *G. glabra* L.) were added to 14 L of an aqueous 80% ethanol solution. The mixture was stirred for 4 h at 70 °C and filtered through a sintered glass funnel. The supernatant was recovered and concentrated under vacuum to dryness. The resulting crude extract (175.3 g) was analyzed by HPLC (see Section 2.10) to determine the GL content.

2.8. Influence of extractant and displacer concentrations on the extraction profile of GL from a crude extract of licorice roots

Several extractant/GL molar ratios (n_{Al336}/n_{GL}) (3; 11; 22; 44) were tested. For each condition, 3 g of the crude licorice root extract (0.46 mmol of GL) was dissolved in 20 mL of the mobile phase and maintained at pH 7. The aqueous mobile phase was pumped in the descending mode at 20 mL/min with a column rotation speed of 1000 rpm. During this extraction step, anionic and neutral lipophilic compounds were retained by the stationary phase while cationic and neutral hydrophilic compounds were eluted. The mobile phase was then supplemented with the displacer KI. Different extractant/displacer (n_{Al336}/n_{KI}) molar ratios (2;1) were also tested.

2.9. Influence of increasing crude extract sample loading mass

The crude extract of licorice roots (3 g, 10 g, and 20 g) was dissolved in 20 mL, 100 mL or 400 mL of the aqueous mobile phase, respectively. The mobile phase was progressively pumped from 0 to 20 mL/min in 2 min to maintain the hydrodynamic equilibrium inside the column. Fractions were collected every minute.

2.10. TLC, HPLC and MS experiments

All fractions were checked by TLC on Merck 60 F_{254} silica gel plates, developed with $CHCl_3$/MeOH/water (70/30/2.5, v/v/v) and using 50% sulfuric acid in EtOH as spray reagent. Quantitative analyses were performed on a Waters HPLC system (Saint-Quentin, France) equipped with a 600E pump, a 717plus autosampler and a Jasco CO965 column oven. The chromatographic column (Luna, 250 mm × 4.6 mm, 5 μm, Phenomenex, Le Pecq, France) was maintained at 22 °C. The mobile phases were 0.025% TFA in water (solvent A) and acetonitrile (solvent B). The separation was performed at 1 mL/min with a gradient elution starting with 80% solvent A for 10 min. Then solvent B increased to 60% in 40 min, to 100% in 1 min and was maintained for 7 min. The injection volume was 15 μL. The system was coupled to a Waters® 996 photodiode array detector monitored at 252 nm. Data acquisition was controlled by the Empower 2 Software (Waters). Calibration curves were established by serial dilution of three independent stock solutions (0.1, 0.5, 1, 1.5, and 2 g/L) and by plotting the peak area recorded from HPLC chromatograms as a function of GL concentration. The identity of GL in the crude extract of licorice roots was confirmed on the basis of retention time and compared with the corresponding standard.

Mass spectrometry experiments were carried out to check the fractions of interest and to identify the major compounds present in the licorice crude extract together with GL. Samples were directly infused in a quadrupole time-of-flight hybrid mass spectrometer (QTOF micro®, Micromass, Manchester, UK) equipped with an electrospray source. The mass range of the instrument was set at m/z 100–1650 and scan duration was set at 1 s in the positive and negative ion modes. The capillary voltage was 3000 V, the cone voltage was 35 V, and the temperature was 80 °C.

3. Results and discussion

3.1. FCPE300® characteristics

The volume of the FCPE300® column was measured as 303.5 ± 1.3 mL. Fig. 4 represents the percentage of the stationary phase retention inside the FCPE300® partition cells as a function of flow rates for different biphasic solvent systems. After biphasic system equilibration, a low and constant pressure drop (ΔP = 2–6 bars) was measured in the different operating conditions. A linear relationship between the flow rate and the stationary phase retention was observed for all of the solvent systems tested. This linearity is in accordance with previous results obtained on CPC systems [26]. The intercepts of the linear regression curves (≈76%) with the ordinate axis, defined as the theoretical retention of the stationary phase at zero flow, allowed the calculation of the dead volume of the column. This volume was 73 mL (24% of the total column volume). A stationary phase retention higher than 50% was obtained when working up to 80 mL/min for the six solvent systems tested (Fig. 4). For the *n*-heptane/water system, which displayed the highest stability, the stationary phase retention was 72% at 50 mL/min and still 55% at 300 mL/min. For the less stable *n*-butanol/water system, the stationary phase retention was 61% at 50 mL/min, 26% at 200 mL/min and only 9% at 300 mL/min, indicating poor retention when working at flow rates higher than 50 mL/min. For the intermediary *n*-heptane/MeOH, EtOAc/water, M*t*BE/water and EtOAc/*n*-butanol/water solvent systems, the stationary phase retention was maintained higher than 50% when working up to 100 mL/min. However, when increasing the flow rate to 200 mL/min, the stationary phase retention was 52% for the M*t*BE/water solvent system, and only 29% for the EtOAc/*n*-butanol/water solvent system. For experiments carried out in the ascending mode, the stationary phase retention when using the *n*-heptane/water system was 71% at 50 mL/min and decreased to 46.6% at 300 mL/min. With the EtOAc/*n*-butanol/water solvent system, the stationary phase retention was 45.9% at 100 mL/min and only 14.3% when increasing the flow rate to 200 mL/min. The slope of the different linear regressions indicate that the FCPE300® column can work at flow rates ranging from 10 to 100 mL/min for the most common biphasic solvent systems.

3.2. Influence of flow rates on the extraction profile of GL

Our objective was to find the optimum flow rate to achieve a short and efficient extraction of GL. Increasing the flow rate leads to a better process efficiency and shorter separation, but it reduces the stationary phase retention (and thus the extractor capacity) especially in the presence of amphiphilic species [27]. The biphasic solvent system EtOAc/*n*-butanol/water (3/2/5,

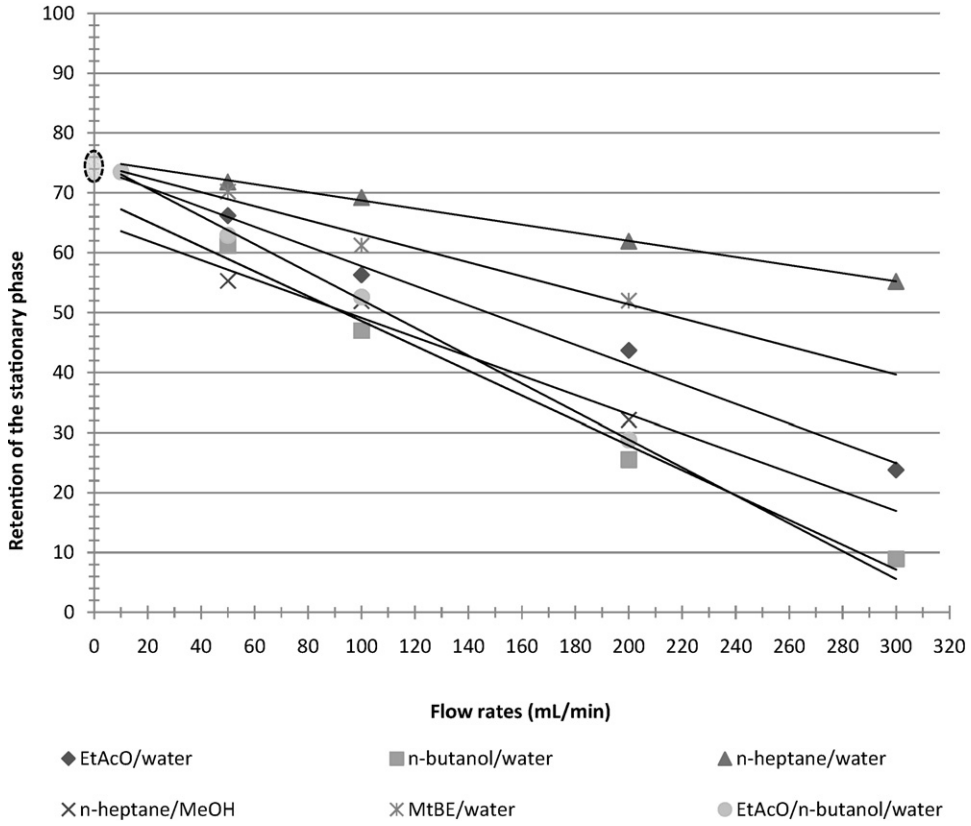

Fig. 4. Influence of flow rates on the stationary phase retention for different biphasic solvent systems at a rotation speed of 1000 rpm. SF: retention factor (%); intercept: (*y*-axis with convergent lines) = volume of connecting ducts (24% of total column volume).

v/v/v) and the extractant Al336 were selected on the basis of a previous method developed for the purification of glucosinolates (another class of ionized heterosides) by ion-exchange centrifugal partition chromatography [11]. This choice was validated by qualitative GL partition coefficient measurements by TLC. The extraction profiles of 200 mg GL standard were examined at 10, 20, and 50 mL/min. As illustrated in Fig. 5 and Table 1, working at 10 mL/min allowed a 100% recovery of GL after 75 min. In this condition GL

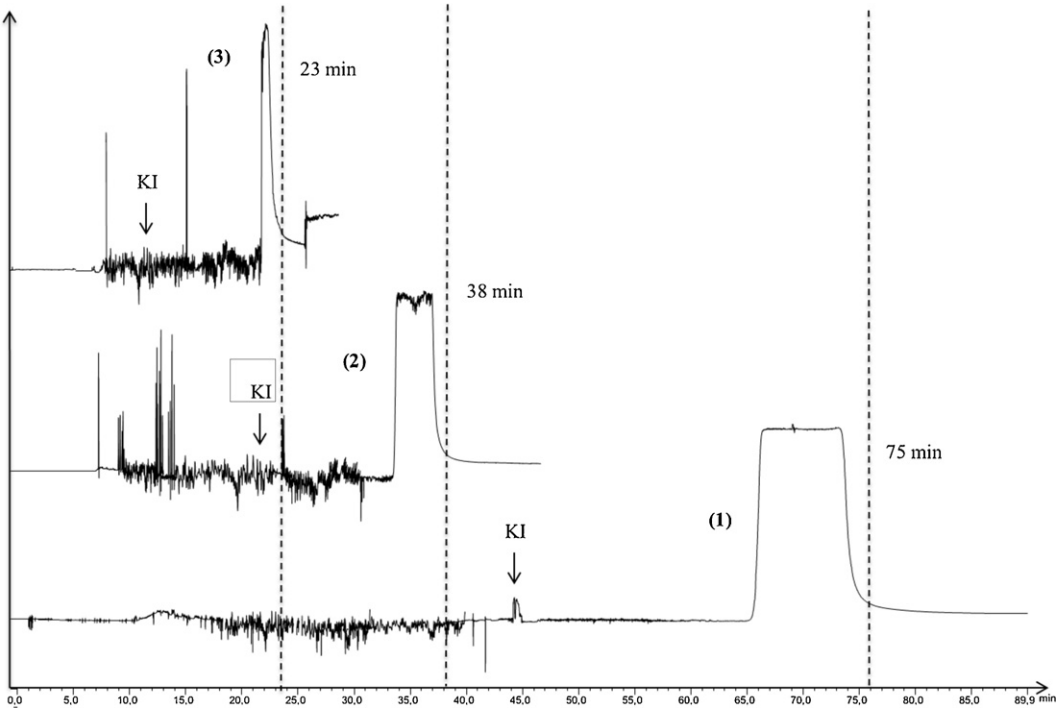

Fig. 5. Influence of the flow rate on the extraction profile of GL. (1) 10 mL/min, (2) 20 mL/min and (3) 50 mL/min. Experimental conditions: rotation speed = 100 rpm; loading of 200 mg GL standard; $n_{Al336}/n_{KI} = 1$; $n_{Al336}/n_{GL} = 7.4$; UV absorbance monitored at 252 nm. Dash line = end of GL back-extraction.

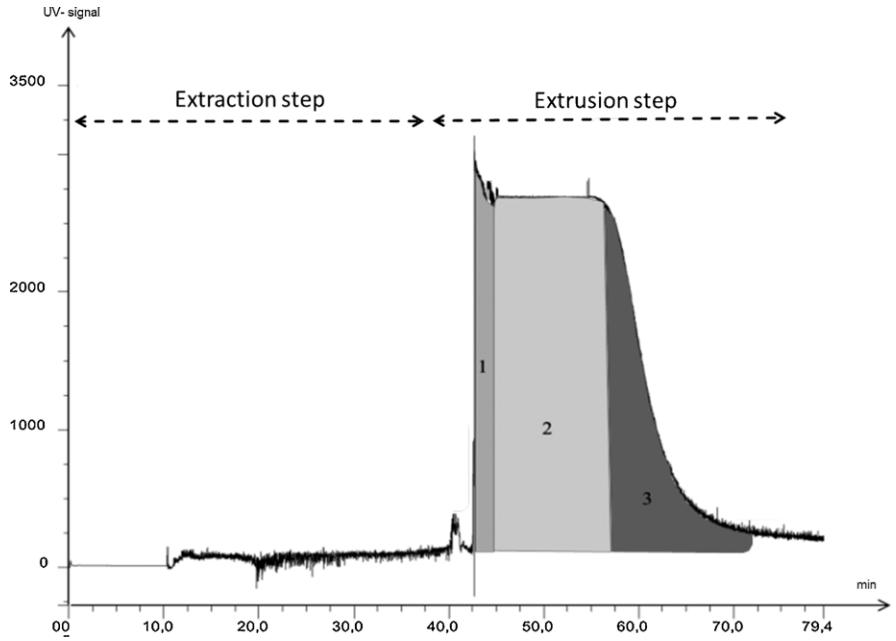

Fig. 6. Concentration profile of GL inside the FCPE300® column. Loading of 131 mg of GL standard. The organic stationary phase was extruded by pumping the aqueous mobile phase in the descending mode at 10 mL/min. Rotation speed: 1000 rpm. Zone 1: first fraction extruded; zone 2: constant GL concentration; zone 3: gradual decrease of GL concentration.

was back extracted during 7.5 min (outlet concentration = 3.2 mM) and the stationary phase retention was 67.2% (experiment 1). At 20 mL/min, a total GL recovery was possible in 38 min and back extracted in a total of 3.5 min (outlet concentration = 3.1 mM). The stationary phase retention was 64.9% (experiment 2). At 50 mL/min, total GL recovery was complete after 23 min and back extracted during 1.5 min (outlet concentration = 3.1 mM). The stationary phase retention was 62.4% (experiment 3).

The reduction of the total IP-CPE process duration is proportional to the increased flow rate from 10 to 20 mL/min, but not from 20 to 50 mL/min. This could suggest a kinetic limitation during the back extraction of the retainer (Cl^-). These results highlight the capacity of the FCPE300® to work at high flow rates while maintaining high retention of the stationary phase and the compressive character of the shocklayers, *i.e.* thin analyte border zones in the isotachic train. This feature is interesting since the flow rates represent a major limiting factor in support free liquid–liquid separation systems [10,28].

Due to the chemical complexity of licorice root composition and due to the surfactant character of GL [29,30], we decided to fix the flow rate at 20 mL/min when applying the process to the crude extract of licorice roots. In fact, working at 20 mL/min offers the best compromise in terms of extraction process duration, GL outlet concentration, and stationary phase retention.

3.3. Concentration profile of GL in the FCPE300® partition cells before the back extraction step

The longitudinal concentration profile of GL inside the FCPE300® column after extraction in the organic stationary phase, without any back extraction process, was determined using the extrusion procedure. The column was filled with the organic stationary phase containing 3.3 mM, 10 mM or 30 mM of Al336. In each experiment 200 mg of GL standard (0.238 mmol) were injected. When working with 10 mM or 30 mM of the extractant Al336, no GL exited from the column, indicating that all GL was retained in the stationary phase by the extractant. When working with 3.3 mM of Al336, an excess of GL (69 mg) was recovered out of the column, indicating that only 131 mg (0.156 mmol) of GL were effectively extracted. Considering that the retention volume is 204 mL at 10 mL/min, the amount of extractant inside the column is 0.67 mmol. We can conclude that 0.67 mmol of Al336 are necessary to achieve the complete extraction of 0.156 mmol of GL. A minimum molar ratio $n_{Al336}/n_{GL} = 4.3$ is thus necessary to quantitatively extract GL. As illustrated in Fig. 6, the extrusion profile of GL was characterized by three distinct zones.

The first 20 mL extruded from the column contained the greatest amount of GL (Fig. 6, zone 1). This indicates that the amount of GL is higher in the first partition cells at the head of the column. The fractions recovered during the subsequent 12 min (120 mL) contained a constant amount of GL (Fig. 6, zone 2), reflecting a homogeneous partition of GL inside the column. Finally a gradual decrease in GL concentration was observed during the last 10 min (100 mL) of the extrusion process (Fig. 6, zone 3). This could be due to dispersion phenomena between the stationary phase containing the extractant-GL ion pairs and the freshly pumped mobile phase.

3.4. Influence of the inlet sample volume

As in CPC systems, sample loading is a crucial step in centrifugal partition extraction. Indeed, the sample can disrupt the hydrodynamic equilibrium of the biphasic solvent system [31]. This disruption may result from the nature of the sample (viscosity, surfactant properties), its concentration or from the injection procedure. As shown in Fig. 7, when 131 mg of GL standard are loaded in 20 mL (7.8 mM) of the aqueous mobile phase, all GL was recovered in a total of 66 mL (outlet concentration = 2.4 mM). When the same quantity of GL standard was directly pumped with 1 L (0.156 mM) or 2 L (0.078 mM) of the aqueous mobile phase, GL was recovered in a total of 56 mL (outlet concentration = 2.8 mM) and 48 mL (outlet concentration = 3.2 mM), respectively.

The outlet concentration of GL was significantly increased as a function of the feed volume. This concentration effect obtained by displacement processes has already been described [32]. In all systems based on affinity chromatography or more precisely on displacement processes, the action of the displacer enables the solutes

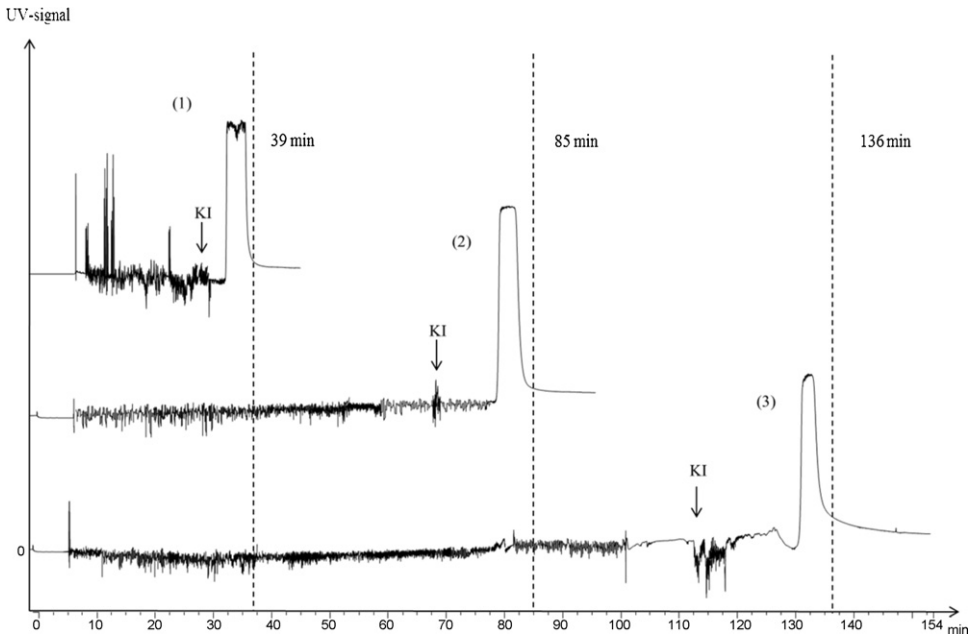

Fig. 7. Influence of the inlet sample volume on the quality of GL extraction. (1) 20 mL; (2) 1 L and (3) 2 L. Loaded sample mass 200 mg of GL standard; flow rates = 20 mL/min, rotation speed = 1000 rpm. $n_{Al336}/n_{KI} = 1$; $n_{Al336}/n_{GL} = 4.2$; UV absorbance monitored at 252 nm. Dash line = end of GL back-extraction.

to exit from the column at high concentration due to competition for the adsorption sites in the stationary phase. This phenomenon is interesting for the analysis of contaminants which are generally present at low concentrations in high volumes of a liquid phase or for the separation of critical trace elements such as in proteomic applications [32]. Here the results reflect the capacity of the extractor to pump large volumes of the mobile phase with very low analyte concentrations without affecting the formation of lipophilic ion pairs between the anionic analytes and the extractant. This also indicates that the volume of the feeding phase only affects the overall duration of the extraction process.

3.5. Influence of the ratio extractant/displacer on the quality of GL extraction starting from a crude extract of licorice roots

The amount of GL in the crude extract of licorice roots was 12.8% on a dry weight basis, as determined by HPLC analyses. A large number of secondary metabolites have been identified in licorice roots, among which more than 24 saponin derivatives, 45 flavonoid derivatives and 18 phenolic compounds [15,30]. All these compounds are potentially ionisable and thus can interact competitively with the extractant. In this section, influences of n_{Al336}/n_{KI} and of n_{Al336}/n_{GL} on the quality of GL extraction were investigated. The extraction profiles obtained for each experiment (see Table 2) are given in Fig. 8.

As a minimum molar ratio $n_{Al336}/n_{GL} = 4.3$ is necessary to fully extract standard GL, we used 12 mM Al336 (stationary phase retention at 20 mL/min = 197 mL) and a ratio $n_{Al336}/n_{KI} = 1$. In these conditions a significant quantity of GL was lost, as confirmed by TLC and HPLC analyses of the first collected fractions. This indicates that all GL was not captured by the extractant and that the exchange sites available for GL were insufficient. As a result, an isotachic train profile without clearly defined concentration plates was obtained (Fig. 8, profile 1). This loss of GL was attributed to the presence of other ionic compounds naturally present in the crude extract and competitively retained by the extractant in the stationary phase. When increasing the extractant concentration up to 48 mM, GL was fully retained and further back extracted, as shown by the characteristic back extraction profile in the form of a well-structured isotachic train. However, the residence time of GL inside the column was too long (60 min, Fig. 8, profile 2). When reducing the displacer concentration (ratio $n_{Al336}/n_{KI} = 20.5$, $n_{KI}/n_{GL} = 1$), the separation of GL from the other analytes was improved but the global process duration was dramatically increased, thus decreasing the productivity of the process (75 min, Fig. 8, profile 3). The optimum molar ratios were found to be $n_{Al336}/n_{GL} = 13$ (Al336 concentration = 31 mM) and $n_{Al336}/n_{KI} = 1$. In these conditions, the amount of extractant was high enough to recover all GL and provided efficient separation of GL from the other major flavonoids present in the licorice crude extract. The equimolar ratio n_{Al336}/n_{KI} allowed the reduction of the total process time to only 37 min (Fig. 8, profile 4).

3.6. Increasing mass sample loading

The objective here was to investigate the ability of the FCPE300® to achieve the extraction of GL when loading up to 20 g of the crude licorice root extract. The stationary phase retention was

Table 2
Experimental conditions for the influence of extractant and displacer concentrations on the extraction profile of GL from 3 g of crude extract of licorice roots.

Experiment	C_{Al336} (mM)	Molar ratio (n_{Al336}/n_{KI})	Molar ratio (n_{Al336}/n_{GL})	Total process time (min)	Analyte back extraction (min)
1	12	1	5	40	8.3
2	48	1	20.5	60	7.6
3	12	25.5	5	75	15
4	31	1	13	37	5.6

GL concentration was estimated at 0.46 mmol; C_{Al336}: extractant concentration; C_{KI} = displacer concentration; n_{Al336}/n_{KI} = extractant/displacer molar ratio; n_{Al336}/n_{GL}: extractant/GL molar ratio. See Fig. 8 for the effect n_{Al336}/n_{KI} and n_{Al336}/n_{GL} molar ratios on the quality of GL extraction.

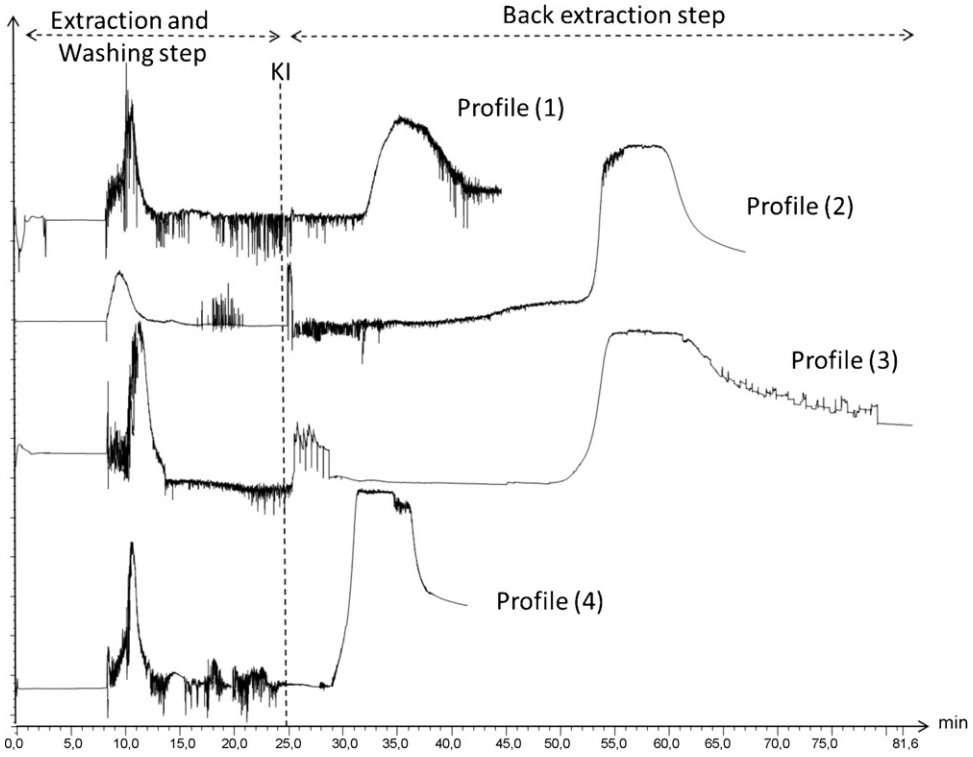

Fig. 8. Influence of extractant/displacer (n_{Al336}/n_{KI}) and extractant/GL (n_{Al336}/n_{GL}) molar ratios on the quality of GL extraction. Flow rate: 20 mL/min; rotation speed: 1000 rpm; loading of 3 g of the crude extract of licorice roots. From profile (1) to profile (4): experiments with various ratios (n_{Al336}/n_{KI}) and (n_{Al336}/n_{GL}) (summarized in Table 2). UV absorbance monitored at 252 nm; dash line = start of back-extraction step.

reduced from 46.1% to 43.6%, 39.4% and 0% (total flooding of the stationary phase) when increasing sample mass from 3 g to 10 g, 20 g and 25 g, respectively. This phenomenon, previously observed for the purification of heparins [33] by ion-exchange CPC, is probably accentuated by the surfactant character of GL. As the outlet concentration of GL is directly correlated to the displacer concentration, we decided to reduce the KI concentration by a factor of 2 (molar ratio $n_{Al336}/n_{KI} = 2$). In this way, the back extraction of GL and other analytes was more progressive. The extraction profile of GL obtained by injecting 20 g of the crude extract is presented in Fig. 9. A 45 min extraction/washing step was necessary to retain all GL in the stationary phase while eluting all cationic and neutral hydrophilic compounds out of the column.

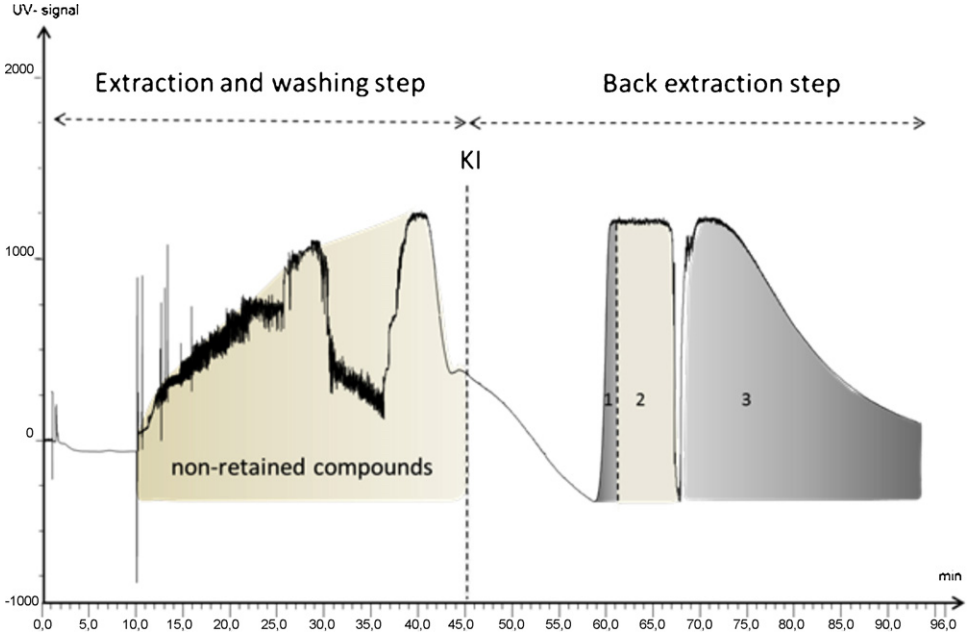

Fig. 9. Extraction profile of GL by IP-CPE, after loading of 20 g of a crude licorice root extract. Flow rate: 20 mL/min; rotation speed: 1000 rpm; $C_{Al336} = 183$ mM; $C_{KI} = 91$ mM. (1): Zone of (liquiritin/isoliquiritin) apioside, (2): zone of GL, (3): zone of the undefined compound. Dash line = start of back-extraction step; UV absorbance monitored at 252 nm.

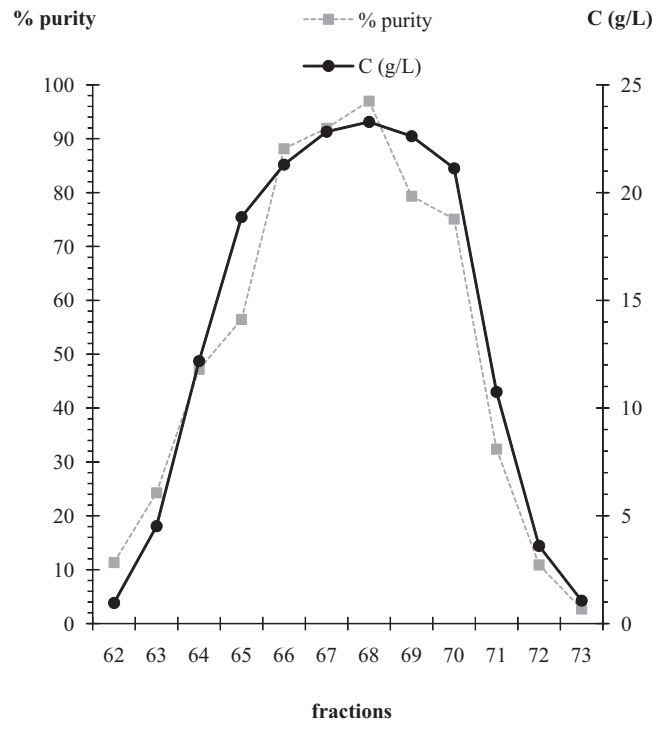

Fig. 10. Fractogram of GL, purity (%) and concentration (g/L).

After the mobile phase was supplemented with the displacer (KI), we observed the formation of an isotachic train which could be divided into 3 zones: the first zone (from 59 to 61 min, *i.e.* 40 mL) corresponded to the back extraction of a major flavonoid of licorice roots. MS analyses revealed a molecular ion at *m*/*z* 589 in the positive ion mode and at *m*/*z* 549 in the negative ion mode, what corresponded respectively to the $[M+K]^+$ and $[M-H]^-$ of liquiritin apioside (or its isomer isoliquiritin apioside). The second zone (from 61 to 69.5 min, *i.e.* 170 mL) corresponded to the back extraction of GL. HPLC analyses revealed that GL was well-separated from the other analytes. In total, 2.21 g of GL were obtained, corresponding to a GL recovery of 86.5%. As shown in Fig. 10, 1.5 g of the GL pool (from fraction 66 to 70) was obtained with a mean purity of 86.3%. The maximum purity was 97% in fraction 68 (240 mg).

The identity of GL was confirmed by MS analyses. Molecular ions were detected at *m*/*z* 861 in the positive ion mode and at *m*/*z* 821 in the negative ion mode, what corresponded to the $[M+K]^+$ adduct and to the $[M-H]^-$ parent ion of GL, respectively. The retention of the stationary phase was 39.4% at the end of the experiment.

These results show that the IP-CPE method allowed the simultaneous extraction and purification of GL from 20 g of a complex licorice root extract in a single run. In comparison, in a recent study dedicated to the separation of GL by counter-current chromatography in the elution mode, the process duration was 350 min to yield 42.2 mg of pure GL from 130 mg of a crude extract of licorice roots with stationary phase retention of 18.1% [12]. The FCPE300® exhibits not only an interesting potential in terms of extraction yield and productivity, but also in terms of purification when working in the ion-pair mode. By introducing exchange sites in the liquid stationary phase, the ion-pair mode results in a succession of extraction/back extraction steps, thus improving separation efficiency.

4. Conclusion

The aim of the present study was to examine the extraction and purification capacity of the lab-scale liquid–liquid FCPE300® extractor when working in the ion-pair extraction mode. This extractor can work at flow rates ranging from 10 to 100 mL/min while retaining high volumes of the stationary phase. By using the IP-CPE method, 2.21 g of the bioactive saponin glycyrrhizin were extracted and purified with good recovery (86.5%) and in one step starting from 20 g of a crude extract of licorice roots. The next step will be to evaluate the robustness and efficiency of the FCPE extractor in other development modes (elution mode, pH-zone refining, etc.) for the extraction of small molecules naturally occurring in complex mixtures and belonging to different chemical classes.

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