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PREPARATIVE SEPARATION OF BERGAPTEN AS A NOVEL COMPOUND FOR THE TREATMENT OF NICOTINISM

1001 PO Session

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Keywords: nicotinism, furanocoumarins, bergapten, psoralen derivatives, memory, depression

Coumarins are a big group of biologically active natural products, known also as important substrates for human cytochrome P-450 2A6 (CYP2A6) and CYP2A13. It was shown that they inhibit CYP2A5-mediated nicotine metabolism *in vivo* in the mice. Hepatic cytochrome CYP2A6 is involved in the 70-80% of the initial metabolism of nicotine and its co-metabolites.

The aim of this study was to investigate whether a known furanocoumarin - bergapten (5-methoxypsoralen) prolongs the behavioral effects of nicotine. Nicotine itself, when administered alone, has the ability to improve memory acquisition and consolidation, as well as exerts antidepressive activity in animal models. These effects are extinguished 48 hours after administration. To investigate the influence of bergapten on the behavioral effects of nicotine the forced swimming test (FST) - animal models of depression, and passive avoidance (PA) test - memory and learning paradigm, were used.

In order to have sufficient quantity of pure bergapten available for pharmacological *in vivo* studies, the dichloromethane extract obtained from fruits of *Heracleum leskovii* Grossh. (Apiaceae) was processed efficiently through high-performance counter-current chromatography (HPCCC) and the process was scaled from analytical to preparative to effect rapid, preparative separation. A two-phase solvent system composed of *n*-heptane/EtOAc/MeOH/H_2O (6:5:6:5, v/v) enables purification of 95 mg of bergapten (purity 99%) after injection of 500 mg of crude extract, in less than 30 min.

Our study revealed that CYP2A6 inhibitor - bergapten (25 mg/kg) prolonged the antidepressive and procognitive effects of nicotine. As nicotine effects were slowed by inhibitors of CYP2A6 this kind of enzymatic inhibition has been proposed as a novel target for smoking cessation, and bergapten may offer a new approach to the treatment of nicotinism.

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FRACTIONATION OF BISDESMODIC SAPONINS WITH ANTI-TUMOR ENHANCING ACTIVITY FROM *SAPONARIA OFFICINALIS* BY HPCCC AND USE OF NATURAL CHIRAL SELECTORS AND MONITORING BY OFF-LINE ESI-MS/MS INJECTIONS

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Session IV

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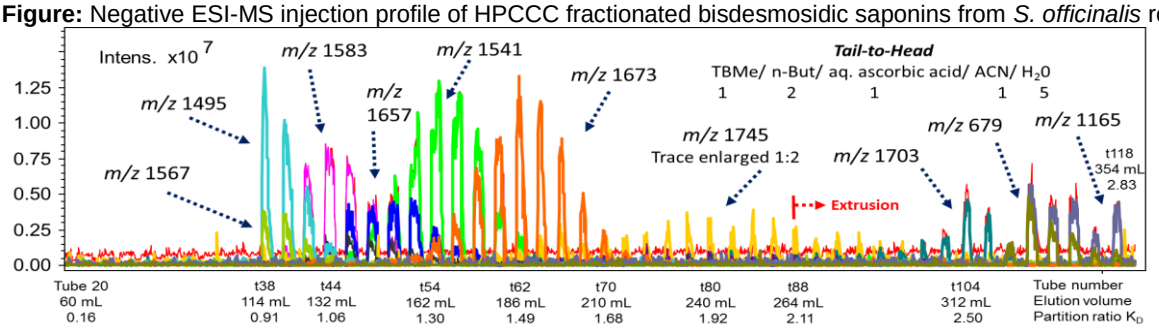
Keywords: Saponaria officinalis, polar anti-tumor saponins, preparative ESI-MS/MS profile

Saponaria officinalis L. (Caryophyllaceae) (soapwort) is a medicinal plant known from Europe to Central Asia. Quillaic acid and gypsogenin bisdesmosidic saponins with specific structural features had been shown to strongly enhance the efficacy of targeted toxins e.g. Sap3-EGF (epidermal growth factor linked to protein toxins) *in-vitro* and *in-vivo* in a synergistic manner (**1**) as a promising novel approach in tumor therapy. Aim of this study was to pre-fractionate and isolate respective target saponins (1300-1800 amu) of similar polarities by HPCCC for further biological evaluation omitting toxic non-volatile reagents. Various biphasic solvent systems with addition of chiral natural selectors had been evaluated to induce specific K_D differences for these saponins (prediction: LC-MS of phases). Strong K_D-value modifying effects on saponins were seen for L-(+)-ascorbic acid (*Asa*). The HPCCC (Spectrum-Dynamic Extractions, column vol. 125 mL) was operated with TBME/ n-butanol/ aq. ascorbic acid (3g–100mL)/ ACN/ H_2O (1:2:1:1:5) (*tail-to-head*, 3.0 mL/min) using *elution-extrusion*. The resulting HPCCC fractions (300 mg injection, t20 - t118) were *off*-line injected in sequence of recovery to an ESI-MS/MS (Bruker HCT Ultra). This combined HPCCC and ESI-MS *guided* isolation procedure (**2**) made use of selective ion traces of the target saponins (cf. Fig.). Elution orders and co-elution effects of minor and major saponins were monitored by selected ion-traces (neg. ESI, *m/z* 100-2500). MS^2 of 5 precursor ions revealed specific sugar substitutions. HPCCC resulted in the fractionation of 9 major saponins, with one very pure saponin (*m/z* 1673.7) (**3**). Saponins with *m/z* 1583, 1673, 1745, 1165 could be directly used for 1D/2D-NMR studies and bio-assays after removal of *Asa*. The mechanism of interaction of *Asa* with saponins could be related to carbonyl-interaction with sugar hydroxy-functions or molecule cavities and seemed not related to molecular weights. Lately, the generation of selected ion trace profile of saponins is a highly versatile tool for accurate tube fractionation and recognition of specific molecules of interest. This method gives access to bio-active saponins for biological evaluation on larger lab-scale.

Figure: Negative ESI-MS injection profile of HPCCC fractionated bisdesmosidic saponins from *S. officinalis* roots.



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METABOLITE PROFILE OF BETALAINS AND FLAVONOIDS FROM *OPUNTIA STRICTA* VAR. *DILLENI BY* HPCCC AND *OFF-LINE* ESI-MS/MS

1004 PO Session

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Keywords: Opuntia stricta var. dillenii, betalain, flavonoid glycosides, ESI-MS/MS metabolite profile

Egyptian *Opuntia* fruits (prickly pears) such as the cultivar *O. stricta var. dilleni* (Cactaceae) were originally spread from Mexico and are hot climate resistant fruits that play an important role as an agricultural crop to enlarge arid areas due to global climatical changes. The profile of secondary natural products, possibly responsible for antioxidant, anti-inflammatory, and anti-cancer effects, and the amount of oligomeric sugars, minerals, vitamins and its high water content classifies *Opuntia* now-a-days as a super-fruit (**1**). Betacyanins and betaxanthins are the responsible pigments for the intensive violet, orange and yellow colors in the *Opuntia* fruits. Betacyanins occur as glycosides with large variations in the substitution with cinnamic acid derivatives (**2**). HPCCC (Spectrum-DE, column 125 mL, 5.0 ml/min, inj. 500 mg, '*head-to-tail*' mode with *elution-extrusion,* TBME/ *n*-BuOH/ ACN/ H_2O (0.7% TFA) [2:2:1:5]) was applied to fractionate the betalains and flavanoid glycosides from a freshly prepared C18 polyphenol fruit extract (**3**). The recovered fractions were injected in sequence to ESI-MS/MS (Bruker HCT Ultra ion-trap, pos. mode, *m/z* 100-1200) to generate a preparative metabolite profile of separated and co-eluting compounds (**2**) using propionic acid in the make-up solvent to omit signal quenching caused by TFA. Selected ion traces visualized that HPCCC well fractionated early eluting pigments (e.g. betanin) and the non-pigment compounds such as flavonoid glycosides. Betanin (*m/z* 551), phyllocactin (*m/z* 637), indicaxanthin (*m/z* 309) and betanidin (*m/z* 389) were recognized as the main pigments, and a minor trace of feruloyl-betanin (*m/z* 727) was found. However, hylocerenin as typical cactus pigment, and organic acids such as piscidic and eucomic acids known from *Opuntia ficus indica* fruits were not detected. This method could be scaled-up for further biological evaluation of these polyphenols.

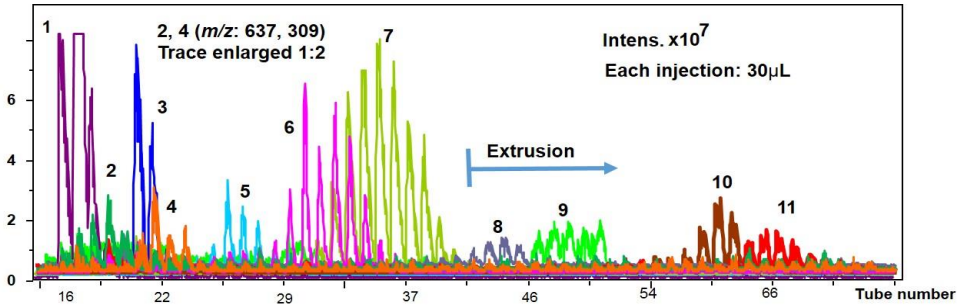

Figure: ESI-MS/MS metabolite injection profile of the HPCCC fractionation of *O. stricta* polyphenol C18 extract from preparative HPCCC coupling with sequential off-line injection to ESI-MS/MS. Selected ion traces of major compounds: **1** betanin (*m/z* 551), **2** phyllocactin (*m/z* 637), **3** not id. (*m/z* 568), **4** indicaxanthin (*m/z* 309), **5** not ident. (*m/z* 689), **6** betanidin (*m/z* 389), **7** isorhamnetin-rutinoside (*m/z* 625), **8** Quercetin-rutinoside (*m/z* 595), **9** not ident. (*m/z* 506), **10** not ident. (*m/z* 620), **11** not ident. (*m/z* 563). One tube fraction represents 5.0 mL CCC elution volume.

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RECOVERY OF THE BETACYANIN CELOSIANIN II AND FLAVONOID GLYCOSIDES FROM *ATRIPLEX HORTENSIS VAR. RUBRA* BY HPCCC AND OFF-LINE ESI-MS/MS MONITORING

1005 PO Session

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Keywords: Atriplex hortensis var. rubra*, celosianin II, flavonoid glycosides, ESI-MS/MS profile*

Atriplex hortensis var. *rubra* (Chenopodiaceae, *engl.* red mountain spinach, red orach) - an ancient vegetable is used for treatment of respiration, digestion and urinary diseases (**1,2**). Rare sulphated flavonoids, kaempferol-, and quercetin-3-*O*-sulphate-7-*O*-α-arabinosides had been elucidated from *A.h.* (**1**). These uncommon structures seem to play a role in plant biological regulation, and possess antiviral and anticoagulant properties (**3**). Preparative HPCCC (Spectrum, Dynamic Extractions U.K., coil vol. 125 mL, *elution-extrusion* approach) was applied using TBME/ *n*-BuOH/ ACN/ H_2O (1/3/1/5; 1% TFA, 4.0 mL/min, *head-to-tail* mode, inj. 460 mg, C18 reversed phase extract) to overview the metabolite profile. The collected fractions were sequentially injected into ESI-MS/MS (Bruker HCT Ultra ion-trap, pos. ESI, *m/z* 100-1200) to monitor target ion traces in the separated fractions (tube 15 – 76) and specific compound co-elutions. To overcome signal quenching caused by TFA, the *make-up* solvent and the tube fractions for MS-detection contained propionic acid. The combination of HPCCC with ESI-MS detection fractionated polyphenols and a principal pigment. The betacyanin 2''-*O*-feruloyl-amaranthine (celosianin II, *m/z* 903*)* was recovered (12 mg)*,* and fractions of flavonoid-glycosides during *elution* and *extrusion*. Quercetin-3-*O*-(malonyl-glc) (*m/z* 551) and kaempferol-3-*O*-(malonyl-glc) (*m/z* 535) were found as main flavonoid glycosides. MS/MS fragmentation of $[M+H]^+$ signals elucidated the potential substitution pattern of flavonoid glycosides by neutral loss differences (Δ*m/z* 86: malonyl; 80: sulphate; 132: arabinosyl, 146: rhamnosyl; cf. Fig.). Major achievement was the recovery of the instable betacyanin celosianin II (*m/z* 903) in a single CCC step which was then fully characterized by 1D/2D-NMR. The HPCCC process induced no pigment degradation as proven by the ESI-MS injection experiment. Selected ion traces clearly guided the accurate process of tube fractionation.

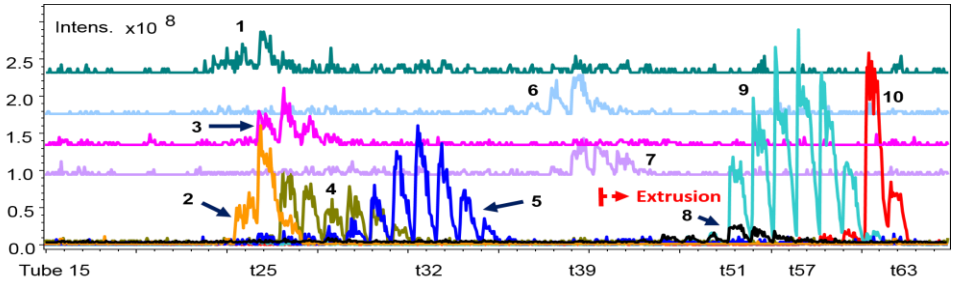

Figure: Sequential *off-line* ESI-MS/MS injections (pos. mode, $[M+H]^+$) of recovered HPCCC fractions with selected ion traces for polyphenol detection in *A. hortensis* leaves (for better visuality, ion traces with graphical off-set were enlarged by a factor 1:3): **1** Kaempferol-ara-SO_4 (*m/z* 499), **2** celosianin II (*m/z* 903), **3** K-glc-malonyl-glc (*m/z* 697), **4** N-feruloyl-putrescine (*m/z* 265), **5** quercetin-rha-glc (*m/z* 611), **6** K-rha-glc (*m/z* 595), **7** Q-ara-glc-malonyl (*m/z* 683), **8** K-ara-glc-malonyl (*m/z* 667), **9** Q-glc-malonyl (*m/z* 551), **10** K-glc-malonyl (*m/z* 535). One tube fraction represents 5.0 mL elution volume. Abbrev.: Q = quercetin, K = kaempferol

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IN-SITU PROTEIN DETERMINATION TO MONITOR CPC CONTAMINATION

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Session VI

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Keywords: Protein contamination, ADCA, CBB, in situ-method

Over the last years, purification of biomolecules by CPC, especially proteins, regained an increasing interest with the development of the Aqueous Two Phase System (ATPS) (1-2). Thereby with the use of proteins, the contamination risk of the equipment is more important. Indeed proteins can easily adsorb on the rotor material. Thus one of the most important problems for use of CPC in the industry is to ensure the cleanliness of the equipment in order to avoid cross-contamination and also the detection of possible protein contamination. These two issues are highly dependent on rotor material. Thus, in this work we studied two different materials: titanium and stainless steel 316. First of all, a cleaning method was improved to remove protein-metal interaction thanks to a pH switch and an organic solvent combination. Then, a direct method that allows the determination of the effective presence of proteins and the extent of contamination is necessary and was therefore developed.

This in-situ method is derived from Amino Density Estimation by Colorimetric Assay (ADECA) (3). It is based on the affinity of a dye: the Coomassie Brillant Blue (CBB) with protonated amino groups. The procedure was optimized to generate this specific affinity with contaminating proteins. A preliminary study was carried out to limit the non-specific adsorption of the dye on the surface. Then, the ADECA method consisted on three steps. First the fixation of the dye to the amino groups was performed. Afterwards, excess dye and non-specific interactions were removed and finally the dye bounded to proteins was eluted by a pH switch. This method was successfully applied to rotors with various extent of proteins contamination (bovine serum albumin). The eluted dye was quantified and found to respond linearly to proteins contamination up to 60mg. Limit of detection and quantification were recorded and depend on the rotor material. Hence, this method should accurately indicate if a full cleaning and sterilization of the rotor is required whatever the material of the rotor. We hope that this work which allows cleaning and in situ determination of proteins contamination will contribute to the development of proteins purification by CPC.

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PROBING THE COMBINATORIAL METABOLOME OF FLAVANOLIGNANS IN MILK THISTLE (*SILYBUM MARIANUM* L.)

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Session

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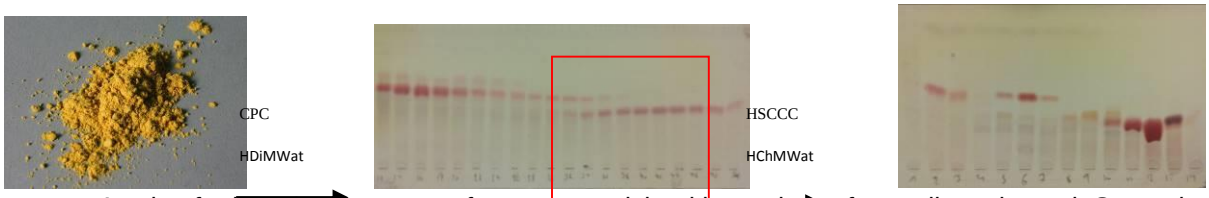
Keywords: Silybum flavanolignan analogues, CPC, HSCCC, orthogonality

Silybum marianum L. (milk thistle) is a medicinal plant that has been used for centuries for liver and gallbladder disorders by protecting liver against snake venom, insect bites, *Amanida phalloides* (mushroom) poisoning, and alcohol abuse [1]. Constituents responsible for the hepatoprotective and anti-hepatotoxicity in standardized milk thistle extract are flavanolignans, which were first characterized in the 1980s and are collectively named "silymarin". Silymarin represents 1.5 to 3% of the milk thistle fruit mass and contains one flavonoid (taxifolin, also known as dihydroquercetin) plus, as reported so far, at least seven flavanolignans (silybin A/B, isosilybins A/B, silychristin, isosilychristin and silydianin) which all share the same molecular weight of 482.44 [2-4]. Our recent preliminary data indicates that *S. marianum* produces a myriad of additional congeneric compounds, tentatively called "*Silybum* Flavanolignan Analogues" (SFAs), with closely resembling structures including stereo- and regio-isomers. Their presence also explains why elucidation of the silymarin complex has been, and continues to be, a phytochemical purification and structural analysis challenge.

As very little is known about the biogenesis and structure of SFAs and their precise biological effects, the objectives of the present work are to (i) develop and optimize new countercurrent chromatography-based methods for the metabolomic fractionation of *S. marianum* extract; (ii) isolate previously unknown SFAs; and (iii) structurally characterize the isolated compounds using MS and 1D/2D NMR. CPC (high capacity) and HSCCC (high resolution) instruments with specifically developed orthogonal two-biphasic solvent systems HChMWat/HDiMWat, (1:22:20:12 (AcOH,0.5%), v/v) were employed. We fractionated the *S. marianum* metaboblome, distinguished the known from the additional compounds, and confirmed that a large number of SFAs remain to be isolated and structurally characterized.



As the further development of new natural health products from silymarin and *S. marianum* depends on the determination and analysis of structure activity relationships of their bioactive constituents, countercurrent separation is likely able to provide new initiatives for the characterization of the *Silybum* flavanolignan metabolome. Furthermore, a better understanding of the metabolomic chemical space of "*Silybum* Flavanolignan Analogues" will provide critical knowledge about the underlying biosynthetic pathways and enhance our ability for their targeted analysis and production.

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COMPARISON OF PARTITION EFFICIENCY BETWEEN SATELLITE AND PLANETARY MOTIONS OF COIL SATELLITE CENTRIFUGE FOR COUNTER-CURRENT CHROMATOGRAPHIC SEPARATION OF 4-METHYLUMBELLIFERYL SUGAR DERIVATIVES

1008
FP
Session X

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Keywords: Coil satellite centrifuge, satellite motion, planetary motion, partition efficiency.

The coil satellite centrifuge (CSC) produces the satellite motion such as the triplicate rotation of coiled column around three axes including the sun axis (the angular velocity, ω_1), the planet axis (ω_2) and the satellite axis (the central axis of the column) (ω_3) according to the following formula: $\omega_1 = \omega_2 + \omega_3$. Improved separation for 4-methylumbelliferyl sugar derivatives were achieved using the common multilayer coiled column with ethyl acetate/1-butanol/water (3 : 2 : 5) for lower phase mobile at the combination of the rotation speeds (ω_1, ω_2, ω_3) = (300, 150, 150 rpm), and (1 : 4 : 5) for upper phase mobile at (300, 100, 200 rpm).

In order to reveal the effect of the satellite motion on the partition efficiency, the peak resolution and the stationary phase retention were measured for each separation with the different rotation speed of ω_2 and ω_3 under the constant revolution speed at ω_1 = 300 rpm. The lower phase mobile produced almost similar results on the peak resolution and the stationary phase retention regardless of the change of ω_2 and ω_3, while the upper phase mobile offered each different result on these two values by varying the rotation speeds of ω_2 and ω_3. Especially, two rotation speeds of ω_2, 147 and 200 rpm, did not retain any lower stationary phase in th coiled column while 150 rpm retains enough volume of stationary phase. On the other hand, the planetary motion providing at (ω_1, ω_2, ω_3) = (300, 300, 0 rpm) or (300, 0, 300 rpm) produced insufficient peak resolution with both lower and upper phase mobiles except (300, 0, 300 rpm) with upper phase mobile, where these results may be caused from the lack of rotation speeds. At lower rotation speed, better partition efficiencies were obtained by the satellite motion than by the planetary motion.

The effect of the hydrophobicity of two-phase solvent systems on the stationary phase retention was further examined using the *n*-hexane/ethyl acetate/1-butanol/methanol/water system at different volume ratios. In the satellite motion at (ω_1, ω_2, ω_3) = (300, 150, 150 rpm), the lower phase mobile obtained almost constant stationary phase retention regardless of the change of the hydrophobicity whereas the upper phase mobile brought different stationary phase retention according to the volume ratio of the two-phase solvent system. However, the planetary motion showed stable stationary phase retention in either phase used as the mobile phase. The overall results indicate that the satellite motion is seriously affected by the combination of rotation speeds and the hydrophobicity of the two-phase solvent system if the upper phase was used as the mobile phase.

DEVELOPMENT OF PRECIPITATION COUTER-CURRENT CHROMATOGRAPHY

1009
FP
Session X

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Keywords: precipitation, solubility, heparin, molecular weight

A (HS)CCC column is typically prepared by winding of PTFE tubing, and there is not any solid support, which enable to separate crude samples. We have developed new separation method for water soluble polymers using this CCC advantage. In this study, heparin was selected as a water soluble polymer. First, heparin was intentionally precipitated in the CCC column using organic solvents. The separated heparin from the solvent would be retained in the column under centrifugal force field. Then, mixture of water and organic solvent was pumped into CCC column as a mobile phase to re-solve the precipitated heparin. If precipitation and dissolution is repeated, the analytes will be separated in order of solubility. We named this method as “precipitation CCC (pCCC)”.

CCC (Type J, column volume 100 mL) was filled with organic solvents, such as methanol, ethanol, isopropyl alcohol or acetone, and rotated at 100 – 850 rpm. Heparin aqueous solution was injected and mobile phase was pumped at 1.0 mL/min. Mobile phase was altered in steps by addition of water to the reservoir containing the organic solvent. Fractionated pCCC samples were subjected to GPC analysis.

Figs 1 and 2 are examples of pCCC chromatograms. The line was represented as the peak area and the dot was retention time obtained by GPC analysis. They were plotted against the retention volume of CCC. When methanol or ethanol was used as solvent, almost of heparin were eluted as solvent front (100 – 150 mL). On the one hand, when IPA or acetone was used, the heparin was strongly hold in the column, and extruded at 250 – 300 mL. Fig 1 shows that heparin was separated into three fractions using MeOH/IPA = 1/3, and the retention time gradually decreased, indicating that heparin was eluted in order of increasing molecular weight. The mixture of heparin and low molecular weight heparin (LMWH) has been successfully separated based on the molecular weight by pCCC method as shown in Fig 2. The pCCC method will be applied to separation of various water soluble polymer according to the molecular weight by choice of solvent combinations and rotation speed.

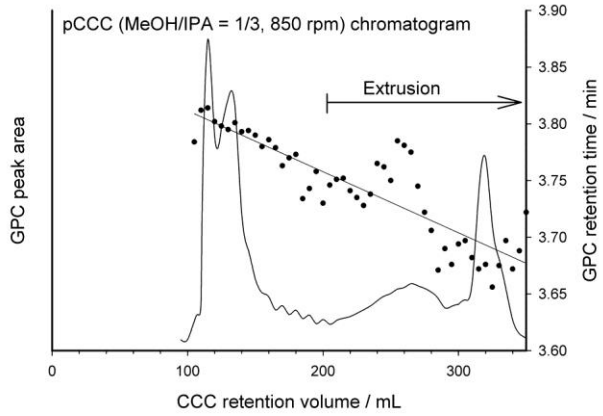

Fig. 1. pCCC chromatogram of heparin

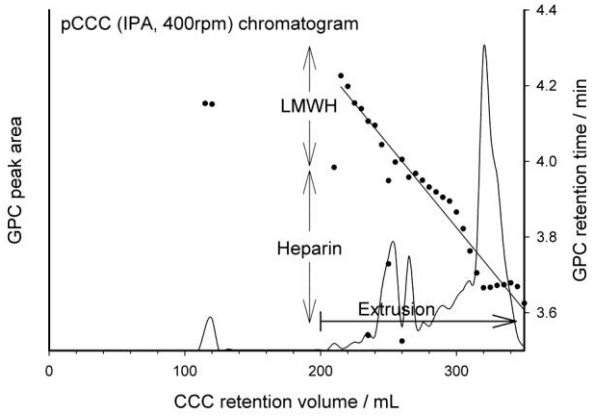

Fig 2. pCCC chromatogram of Heparin & LMWH

ISOLATION AND CHARACTERISATION OF CHLOROPHYLLS AND XANTHOPHYLLS IN GRASS BY A NOVEL SOLVENT SYSTEM USING COUNTERCURRENT CHROMATOGRAPHY

1012
FP
Session IV

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Keywords: chlorophylls, xanthophylls, grass, isolation, novel solvent system

In order to isolate chlorophyll from plant extract by means of High Speed Countercurrent Chromatography (HSCCC) excluding the use of acetone as part of the stationary phase, a novel solvent system composed of hexane/dichloromethane/ethanol/water 4:2:6:2 (v/v/v/v) was applied. The isolation of chlorophylls a, b and pheophytins a, b was successfully performed in the case of grass when dichloromethane was part of the solvent system **(Figure 1/left).** Comparatively, when chloroform was applied as part of the stationary phase, the xanthophyll separation showed better resolution compared to chlorophylls **(Figure 1/right).**

Dichloromethane and chloroform are non-polar solvents. Although, they have similar density (1.49 and 1.32 g/mL), the dielectric constant is higher for dichloromethane (9.1) in comparison with chloroform (4.81). Therefore the polarity of dichloromethane is higher than of chloroform **(1).** Additionally, the Hansen solubility parameters values show that δ P (Polar bonds) and δ H (Polar hydrogen bonding) are meaningfully different. The δ P and δ H values for dichloromethane are 7.3 and 7.1, respectively, whereas for chloroform they are 3.1 and 5.7, respectively **(2).** Consequently, dichloromethane is more polar than chloroform. Therefore, dichloromethane is a polar aprotic solvent and chloroform a non-polar solvent. Therefore, hexane/dichloromethane/ethanol/water 4:2:6:2 (v/v/v/v) is adequate for chlorophyll separation because it changes the system to yield more polarity. This hypothesis is supported by the fact that the elution mode length was 10 hours and the extrusion mode 2 hours **(Figure 1/left),** whereas with chloroform the elution mode length was 7 hours and the extrusion mode 4 hours. Structure elucidation of chlorophylls, pheophytins and xanthophylls was done by modern spectroscopy techniques including LC-APCI-MS/MS and Nuclear Magnetic Resonance (NMR) 1D/2D-NMR experiments (^{13}C, $^{1}H/^{1}H$-COSY, HSQC, HMBC) **(3).**

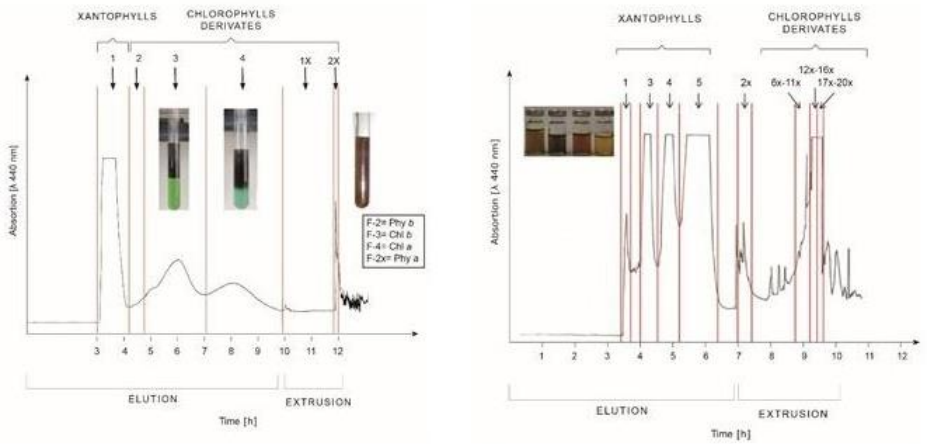

Figure 1. HSCCC chromatogram from grass extract with elution and extrusion mode. **Left**: solvent system: hexane/dichloromethane/ethanol/water (4/2/6/2). Chlorophyll separation showed better resolution in comparison to xanthophylls in elution and extrusion mode. **Right**: solvent system: hexane/chloroform/ethanol/water (4/2/6/2). Xanthophyll separation showed higher resolution in elution in comparison with chlorophylls in extrusion mode.

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SEPARATION OF ISOMERIC MONOSACCHARIDES BY RECYCLING ELUTION-EXTRUSION COUNTERCURRENT CHROMATOGRAPHY

1013
FP
Session II

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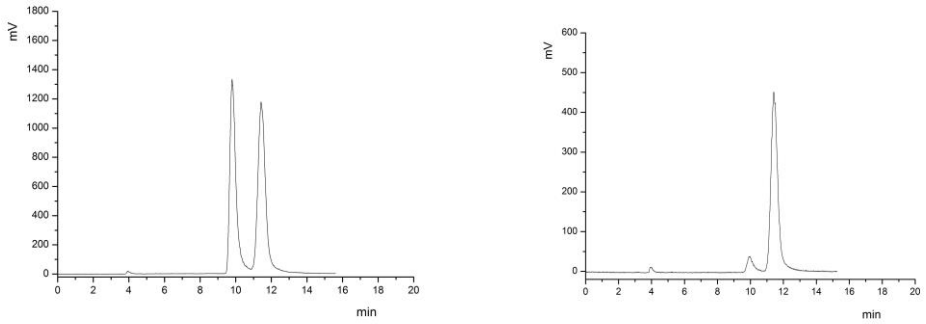
Keywords: Isomeric monosaccharides; Elution-extrusion countercurrent chromatography; Separation

Monosaccharides are difficult to separate and analyze by conventional methods. Traditionally, precolumn derivatization is necessary for separation of monosaccharides by liquid chromatography or gas chromatography due to their high polarity. Elution-extrusion counter-current chromatography is especially useful for separation of components, in which sufficiently large differences in polarity are involved based on their chemical structures. However, in our present work, elution-extrusion countercurrent chromatography was investigated for separation of two isomeric monosaccharides, including fructose and glucose. Both of them are highly polar and soluble in water. Generally, aqueous-aqueous solvent system could be selected for separation of monosaccharides, but here the biphasic solvent system water-butanol (1:1, v/v) was selected, in which a very low partition coefficient of <0.1, was observed for both monosaccharides. The aqueous phase was used as the stationary phase and the organic phase was used as the mobile phase. It was impossible to elute the monosaccharide from the stationary aqueous phase by conventional elution method if organic phase was used. Therefore, recycling elution mode was used after the sample was injected. Almost baseline separation could be achieved for the two monosaccharides by recycling elution-extrusion chromatography, as shown in the HPLC analysis (Figure 1). A mathematic model for separation of isomeric monosaccharides by recycling elution-extrusion countercurrent chromatography was proposed, in which recycling time, extrusion time and peak resolution would be discussed.



(a) (b)

Figure 1. HPLC chromatograms of (a) two isomeric monosaccharides and (b) fractions from modified elution-extrusion counter-current chromatography.

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PRODUCTION OF THREE NEW ANTIOXIDANTS FROM EDELWEISS BY MULTI-HEART CUTTING CPC-LC

1014 PO Session

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Keywords: Edelweiss, antioxidants, CPC-LC, prepLC, scale-up

Leontopodium alpinum, 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 several diseases such as bronchitis or cancer. Wild Edelweiss is protected by the law but the plant is now cultivated in large numbers and extracts of the aerial parts are used for their anti-oxidative properties (1,2). In order to improve clinical research and provide high quality standards, the production of three new antioxidants from Edelweiss plant is required, namely: leontopodic acid A, leontopodic acid B and 3,5-dicaffeoylquinic acid. HPLC analysis exhibits the complexity of this plant extract (1,2). Unfortunately, the compounds of interest have very close chemical structures which prevents their isolation by HPLC at preparative scale due to insufficient resolution. The CPC technique was found to be the unique complementary technique to LC in order to combine two different selectivities through two different mechanisms of separation.

As the CPC separation did not provide sufficient resolution for the separation of the three compounds of interest, the technique was combined to LC technique to recover the antioxidants from Edelweiss plant with the required purity. Thereby, the CPC technique led to a first separation of the three compounds according to their partition coefficients in the solvent system and the LC technique was performed on the recovered fractions to lead to a second separation. The multi-heart cutting CPC-LC was enforced at laboratory scale which represents an innovative separation for the purification of the Edelweiss plant. To produce these three antioxidants, the CPC and LC methods were then transferred at industrial scale. The separation qualities were preserved. Up to two gram of the full Edelweiss plant extract was injected and the three antioxidants were recovered with the required purity, as checked by analytical HPLC and MS.

The complementary use of CPC and LC techniques for the production of three new antioxidants from Edelweiss plant is a strategic combination which represents an innovative separation. This multi-heart cutting CPC-LC allows a gain of time and helps recovering the antioxidants with a high purity. The implementation of the multi-heart cutting CPC-LC method for preparative purposes in industry is achieved.

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CARNOSOL PURIFICATION FROM ROSMARINUS OFFICINALIS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY, FROM LABORATORY TO INDUSTRY

1015 PO Session

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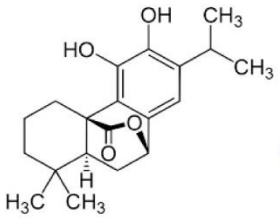
Keywords: rosemary, scale-up



Rosemary (*Rosmarinus officinalis*) is an aromatic herbal plant belonging to the Lamiaceae family and known for its medicinal and taste properties. Recent studies have shown its pharmacologic activities for cancer chemoprevention and therapy due to phenolic compound presence such as carnosol, carnosic acid and rosmarinic acid. **Carnosol** was more specifically evaluated for anti-cancer properties in prostate, breast, skin, leukemia and colon cancer showing promising results. Its purification is required at lab-scale for toxicology studies and at industrial scale for production as an active ingredient. In this context, we present the centrifugal partition chromatography (CPC) method development and carnosol purification from a Rosemary leaves extract on a lab-scale instrument, highlighting the advantages of the CPC technique on natural products purification.

After a rapid method development on a small-scale 35-mL CPC instrument that allowed for the determination of the solvent system and maximum sample concentration and volume, the purification was transferred on an industrial-scale 1-liter instrument using the "free space between peaks" method. The method takes into account the technical limitations of the larger instrument, such as pressure and/or maximum centrifugal field, and allows, by simply running an analytical-sized injection on the large scale rotor, to give an accurate prediction of the maximum sample load and best throughput. The 0.27 g of rosemary extract maximum load on the 35-mL CPC was transferred as 9 g load (33 times bigger load) on the 1000-mL CPC (28 times larger column volume). If the scaling-up in CPC instruments is not directly homothetic, the described protocol makes it highly predictable through few simple experiments.

RECOVERY OF ANTIBACTERIAL CYSTOBACTAMIDS FROM *CYSTOBACTER* SP. BY HPCCC AND OFF-LINE ESI-MS/MS METABOLITE PROFILING

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FP
Session II

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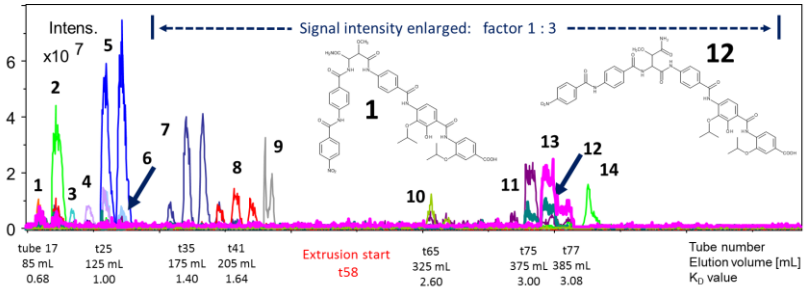
Keywords: Cystobacter sp., cystobactamide, antibiotics, preparative ESI-MS/MS profile

Myxobacteria are known for the production of unique antibacterial antibiotics, such as the myxo- and corallopyronins and the disciformycins (**1,2,3**). Recently, the cystobactamids, which constitute a new class of myxobacterial topoisomerase inhibitors with strong broad-spectrum activity against Gram-negative multiresistant pathogens, have been discovered (**4**). This compound class is of great interest for development as a remedy to combat the multiresistant human pathogens. However, downstream procession is hampered by the fact that enrichment of cystobactamids from crude extracts via flash-chromatography leads to substantial loss of compounds and an economical process for their recovery remains to be established. In this study we have investigated HPCCC as a novel method for the recovery of cystobactamids from crude extracts of the producer *Cystobacter* sp. (**4**). Our model substances for the preparative fractionation were the two cystobactamids (M_r 919-1 and 919-2, structures cf. Fig. - ion signals **1** and **12**, ESI neg. $[M-H]^-$ *m/z* 918) showing minimum inhibitory concentrations in the low µg/mL range (**4**). Suitable solvent systems for recovery were evaluated by LC-ESI-MS analysis. Respective K_D-values of targets in the phase layers were used for prediction of compound elution and occurrence of potential co-elution effects with other metabolites. The HPCCC (Spectrum-DE, column vol. 125 mL) was operated with *n*-hexane/EtOAc/MeOH/H_2O (1:2:1:2) (*head-to-tail, flow* 5.0 mL/min) using *elution-extrusion* (start at tube 58). The resulting HPCCC fractions from a 100 mg sample (tube 17-97) were injected *off*-line in sequence of recovery to an ESI-MS/MS (Bruker HCT Ultra) to project a mass spectrometry metabolite profile (**5**), and the target cystobactamids based on selected ion traces (neg. ESI, *m/z* 100-2500). (cf. Fig.). Elution orders and co-elution effects of minor and major congeners were monitored and MS^2 of 5 precursor ions delivered fragment ions for structural identification and confirmation. HPCCC resulted in the fractionation of the two principal cystobactamids visualized by selected ion traces (ESI neg) with low signal intensity in close eluting tubes (t17-t19 and t53-57). Major ion signals of good purity were seen for metabolites **5**, **7** **8**, **9**, **10**, **14** (cf. Fig.). The elution order of the two isobars **1** Mr 919-1, and **12** 919-2 was as expected, but interestingly the HPCCC K_D value of 3.1 for **12** was twice as high as in the LC-ESI-MS K_D prediction. HPCCC removed a majority of metabolites and concentrated the cystobactamids for fine purification steps. As all CCC methods, the recovery process could be extended to larger lab-scale.

Figure: ESI-MS (neg. mode) injection profile with principal $[M-H]^-$ ions of *Cystobacter* metabolites fractionated by HPCCC: *Cystobactamid* 1: *m/z* 918, 2: 372, 3: 475, 4: 371, 5: 403, 6: 417, 7: 260, 8: 387, 9: 363, 10: 221, 11: 590, *cystobactamid* 12: 918, 13: 860, 14: 526. Injection of every 2nd fraction into the ESI-MS.



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FRACTIONATION OF LIPOPHILIC COMPONENTS FROM POTATOES (*SOLANUM PHUREJA*) BY HPCCC AND MONITORING BY *OFF-LINE* INJECTIONS TO APCI-MS/MS

1017 PO Session

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Keywords: HPCCC, APCI-MS/MS, carotenoids, glucocerebroside, cycloartenol

Potatoes, breeding variety Mayan Gold - *(Solanum phureja*) contain a number of lipophilic compounds (unsaponifiable matters). Generally, non-polars such as carotenoid pigments are known to positively influence human health. Intake by food is inversely correlated to the appearance of degenerative diseases such as age-related macular degeneration (**1**). Non-colored membrane components such as sphingolipids play a key-role in eukaryotic cells. Their metabolites, e.g. ceramide, sphingosine-1-phosphate and long chain base phosphates are regulators in apoptotic-like programed cell death (**2**). Aim of this study was to fractionate bioactive metabolites from *S.p.* for spectroscopic analysis (APCI-MS and NMR). A number of biphasic solvent systems had been evaluated by HPLC-DAD to achieve appropriate K_D-values for an optimized separation. The HPCCC (Spectrum-DE, column vol. 125 mL) was operated with *n-hexane/ EtOAc/ EtOH/ H_2O* (5:5:7:4) (*head-to-tail*, 4.0 mL/min) using the *elution - back extrusion* procedure.

The HPCCC fractions (non-polar saponified crude extract: 300 mg injection, t14 – t100) were *off-line* injected in sequence of elution to an APCI-MS/MS (**3**) where 8 principal components were detected. Ion signals at *m/z* 601, 585, 551, and 569 were carotenoid pigments. Violaxanthin (*m/z* 601) was directly recovered and identified by APCI-MS/MS, ^{1}H- and ^{13}C-NMR data. Some further ion signals were tentatively correlated to lutein-epoxide or antheraxanthin (*m/z* 585), and *m/z* 569 for lutein or zeaxanthin. Almost all carotenoid signals gave Δ*m/z* 18 neutral loss cleavages indicating APCI *in-source* fragmentation related to hydroxylation of xanthophylls (**4**). The CCC-fraction amounts were not sufficient in purity for NMR carotenoid identification. The tube 22 (cf. Fig.) contained the glucocerebroside d18:2-C16:0h-Glc as principal compound ($[M+H]^+$ *m/z* 714). The structure was confirmed by APCI-MS/MS (neg./pos.) (**5**,**6**), and 1D/2D-NMR. Further minor concentrated glucocerebrosides of slightly differing substitution were detected in the HPCCC-APCI-MS profile (not displayed here). Tube fractions 95-96 contained the cyclopropane-type steroid cycloartenol (*m/z* 427) confirmed by MS/MS fragmentation (**7**), and ^{13}C-NMR data.

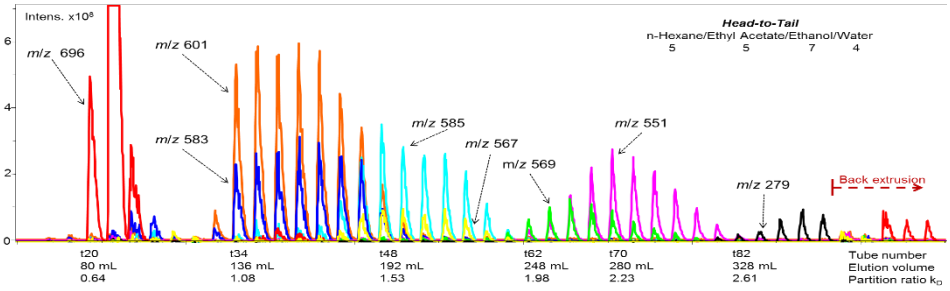

Figure: Positive APCI-MS/MS injection profile of HPCCC fractionated non-polars from *S. phureja* potatoes with selected ion traces $[M+H]^+$. Injection of every second tube fraction.

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VALUE OF *K* FOR APPLICATION OF COUNTER-CURRENT CHROMATOGRAPHY IN THE ISOLATION OF THREE LIPOPETIDE FAMILIES

1018
FP
Session IV

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Keywords: Iturin; Fengycin; Surfactin.

The interest for biological control programs against pests in the world has grown substantially. *In vitro* experiments have shown that the corn-isolated endophytic bacterial strain CNPMS 22 (*Bacillus subtilis*), produces three families of lipopeptides (Iturin, fengycin and surfactin) with antifungal activity [1]. However, the production of these compounds in large-scale requires innovative technologies for their maximum recovery. The counter-current chromatography (CCC) is an efficient technique for the separation and purification of various organic substances. Nevertheless, no lipopeptide separation by CCC has been reported in the literature up to now. In this context, the objective of this study was to evaluate the best solvent system to be used in CCC for separation of three families of CNPMS22 lipopeptides (iturin, fengycin and surfactin), through the assessment of their partition coefficients (*K*). The 9 × 9 map-based solvent selection strategy [2] was chosen to perform those experiments. Initially, 2 mg of the lipopeptide extract, obtained by acid precipitation of the fermentation broth, were added to test tubes. The tested solvents were *n*-hexane-ethyl acetate-methanol-water in the following ratios: (3:7:3:7), (5:5:5:5), (7:3:7:3), (1:9:2:8), (1:9:3:7), (2:8:2:8) and (2:8:3:7) (v/v/v/v), respectively. After the two phase separation, the upper and lower phases were completely separated. The phases were filtered through PTFE filter (0.22µm) and injected on UPLC-ESI-QTOF-MS/MS. The partition coefficients (*k*) were determined with the ratio A_U/A_L where A_U and A_L are the peak areas of the compounds at the upper and lower phase, respectively. The results showed that the ratio of 1:9:3:7 is promising for the separation of iturins and fengycins (Table 1) and the ratio of 5:5:5:5 is the most appropriate for the separation of surfactins (Table 2). This is a first study about solvent system selection for isolation of these three families of lipopeptides to be applied in counter-current chromatography. Hence, further information is required for process optimization and being an applicable method.

Table 1. Value of K for solvent ratio (1:9:3:7)

Compounds	Molecular mass $[M-H]^+$	value of K
Iturin A2	1043.5588	0.2731
Iturin A3-A5	1057.5729	0.5822
Iturin A6-A7	1071.5889	0.6449
Fengycin A	1435.7742	0.0727
Fengycin A	1449.7961	0.1081
Fengycin B	1477.8228	0.7967
Fengycin A	1463.8141	0.2259
Fengycin B	1491.8446	0.7004
Fengycin B	1505.8540	1.1138

Table 2. Value of K for solvent ratio (5:5:5:5)

Compounds	Molecular mass $[M-H]^+$	value of K
surfactin	994.6481	1.6452
surfactin	1008.6709	2.1608
surfactin	1022.6839	2.9309
surfactin	1036.6935	3.2209
surfactin	1044.6576	1.3186

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ENHANCING SILYMARIN FRACTIONATION USING THE CONDUCTOR-LIKE SCREENING MODEL FOR REAL SOLVENTS

1019
FP
Session VI

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Keywords: bioseparations, COSMO-RS, flavonoids, silymarins, nutraceuticals

The market for bio-based products from plant sources is on the rise. There is a global challenge to implement environmentally clean practices for the production of fuels and pharmaceuticals from sustainable resources. A significant hurdle for discovery of comparable plant-derived products is the extensive volume of trial-and-error experimentation required. To alleviate the experimental burden, a quantum mechanics-based molecular modeling approach known as the COnductor-like Screening MOdel for Real Solvents (COSMO-RS) [1] was used to predict the best two-phase solvent system to purify six silymarins from an aqueous mixture. Silymarins are a class of flavonolignans present in milk thistle (*Silybum marianum* L.), which has been used in traditional eastern medicine to treat liver disease. Previous research has shown that these compounds can be fractionated using centrifugal partition chromatography (CPC), but not to an acceptable level of purity [2,3]. Due to previous incomplete fractionation, the silymarins are ideal compounds to assess use of a molecular modeling approach to predict partitioning in a CPC separation. Utilization of the COSMO-RS via the software programs HyperChem, TurbomoleX, and COSMOthermX in order to calculate partition coefficients for compounds in CPC solvent systems was first illustrated by Hopmann et al. [4]. In this study, the methods have been applied to silymarins to evaluate the effectiveness of COSMO-RS in screening solvent systems for separation of biomolecules in a bi-phasic solvent system using CPC. COSMOthermX was used to calculate the activity coefficients of the silymarins in each solvent system, based on the molecular structure of the compounds and phase partitioning data gathered from gas chromatography. The activity coefficient was then used to calculate a partition coefficient for each silymarin in each solvent system. The partition coefficient was verified by experimentation and compared to the results of the model. Use of the COSMO-RS method allowed the range of possible solvent systems to be quickly narrowed down, reducing the quantity of trial-and-error tests and the time required to achieve results.

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TOCOPHEROL SEPARATION WITH DEEP EUTECTIC SOLVENT-BASED BIPHASIC SYSTEMS

1020
FP
Session V

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Keywords: vitamin E, centrifugal partition extraction, water-free solvent system

Tocopherols are a class of molecules which are known for their vitamin E activity. Among these, α-tocopherol is the most important vitamin E source in the human diet. Hence, there is a great interest in separation methods that can provide purified α-tocopherol. The purification of tocopherols involving biphasic liquid systems can be challenging since these vitamins are poorly soluble in water.

In this work, a mixture of tocopherols mainly consisting of α- and γ-tocopherol has been separated using centrifugal partition extraction. For this purpose a biphasic system composed of organic solvents and a deep eutectic solvent (DES) was used. DES are formed when a hydrogen bond acceptor, in this case a quaternary ammonium salt, and a hydrogen bond donor are combined. The mixture has a significantly lower melting point than the pure components and therefore remains liquid at room temperature (1). The tocopherol constituents show different affinity for hydrogen bonding, resulting in different partitioning between the two liquid phases of the solvent system (2). A solvent system screening was performed using the predictive thermodynamic model COSMO-RS to find an appropriate DES-based biphasic system. The most promising biphasic solvent system was selected to perform the separation. For the liquid-liquid chromatographic separation a centrifugal partition extractor (SCPE-250-BIO from Armen Instrument, France) with a column volume of 250 ml was used. Although the DES-rich phase has a higher viscosity than conventional solvents, such as water or methanol, high stationary phase retention was obtained. Approximately 70 % of stationary phase were retained in the column at a flow rate of 20 ml/min and approximately 60 % at 40 ml/min, both at a rotation of 2000 rpm.

The results of this work show the potential of DES-based biphasic systems for the purification of water insoluble natural compounds.

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PROTEIN SEPARATION USING A CENTRIFUGAL PARTITION EXTRACTOR

1021
PO
Session

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Keywords: ionic liquids, aqueous two-phase systems, protein separation

Aqueous two-phase systems (ATPS) provide a mild environment for the separation of biomolecules, such as proteins and peptides, with liquid-liquid extraction or liquid-liquid chromatography (1). Conventional ATPS are composed of two polymers or a polymer and inorganic salt in water. In 2003, Rogers et al. introduced biphasic systems containing imidazolium-based ionic liquids, inorganic salts and water (2). Ionic liquids show high solvation capacity for many compounds. Since proteins partition almost exclusively into the ionic liquid-rich phase, these ATPS are well suited for extraction processes. (3) However, for liquid-liquid chromatographic separations, moderate partition coefficients of the solutes are desirable. Pereira et al. proposed to use ionic liquids as adjuvants to tailor the extraction capacity of biomolecules in polyethylene glycol (PEG)- and salt-based ATPS. Our measurements demonstrate that the addition of ionic liquids significantly influences the partition coefficients of proteins in biphasic systems composed of PEG 1000 and inorganic salts. (3) In these systems the partition coefficients are more suitable for an application in liquid-liquid chromatography. However, it is reasonable to use the minimal possible amount of modifier to tune the partition coefficient to keep the separation and material costs low. The influence of ionic-liquids on protein partitioning in such PEG-based ATPS was determined in shake flask experiments and compared with the influence of sodium chloride, which is commonly used as an additive in biphasic systems for the separation of biomolecules. A mixture of the two proteins lysozyme and myoglobin was separated using PEG-based biphasic systems in a centrifugal extractor (SCPE-250-BIO from Armen Instrument, France).

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RELATIONSHIP BETWEEN THE EFFICIENCY AND ROTATION SPEED IN THE COUNTERCURRENT CHROMATOGRAPHY: SEPARATION OF CYTOTOXIC METABOLITES BY SELECTIVE ENZYMATIC TRANSFORMATION

1022 PO Session

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Keywords: prosaikogenins, rotation speed, stationary phase retention

Countercurrent separation (CS) is one of the efficient methods to separate the pure compound from the diverse natural resources. Establishing an optimal condition is the most important procedure for the efficient separation. The retention of stationary phase (S_f) strongly affects the resolution of peaks. *Du* (1) and *Wood* (2) demonstrate the relationship between the stationary phase retention and various parameters. More recently, Berthod et al. shed light on the relation of resolution and stationary phase retention (3). The rotation speed of countercurrent chromatography machine is also greatly affects the resolution of peaks. With the given solvent system and countercurrent apparatus, the optimal rotation speed was speculated by several preliminary experimental results and calculations. The speculation was further demonstrated by the actual experiments.

Saikosaponins are bioactive compounds from the roots of *Bupleurum falcatum*. Despite of various pharmacological benefits, the application of those compounds is restrained due to their lower bioavailability and the lack of large-scale separation method. The separation method for the metabolites of those compounds was developed with the application of enzymatic transformation. By the enzymatic transformation, glucose at the C-3 position of structure was eliminated. As a result, the polarity and molecular weight were decreased. The converted fraction was then separated by countercurrent chromatography on the preparative scale. Through the investigation on the cytotoxicity of the separated compounds, the converted compounds seem to be more promising for the candidate of anticancer agent.

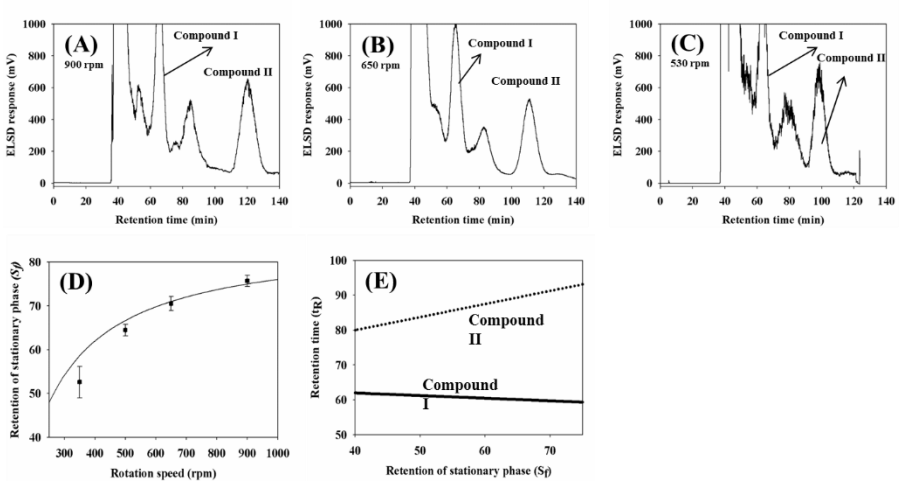

Figure 1. Relationship between the resolution and the rotation speed.

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LINEAR GRADIENT ELUTION IN COUNTERCURRET CHROMATOGRAPHY WITH AVERAGE SPEED OF TARGET COMPOUNDS

1023
FP
Session VIII

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Keywords: linear gradient, average speed, Pulsatilla koreana

Natural products extracts are mixture of compounds of wide polarity range. Gradient elution in countercurrent separation (CS) allows the wide polarity range to be handled and co-eluting compounds to be separated. Several modeling studies (1) have illustrated that various parameters have to be considered for gradient elution. In the present study, solvent systems for the gradient elution were pre-evaluated on the basis of several criteria (e.g. partition coefficients, settling time, and the retention of stationary phase). The linear-gradient change was settled down by speculating average speeds (|*V*|) of target compounds. The important issues in linear gradient elution are the starting point and the duration of the second mobile phase. Those conditions were speculated by the location (*L*) of target compounds.

Pulsatilla koreana (Ranuculaceae) is a perennial herb from the hillocks in South Korea. The root of this plant has long been used as a traditional herbal medicine to treat amoebic dysentery, malaria, chills, and fevers. The major components of *P. koreana* are triterpenoid saponins. Because of their pharmacological activities, a preparative separation method for obtaining pure saponins from *P. koreana* needs to be developed. To date, several triterpenoid saponins of lupane or oleanane-type have been isolated using conventional chromatographic methods despite of low recovery. In the present study, four compounds were separated by linear gradient elution, including hederacolchiside E, which has recently been highlighted as an agent for the treatment of Alzheimer's disease.

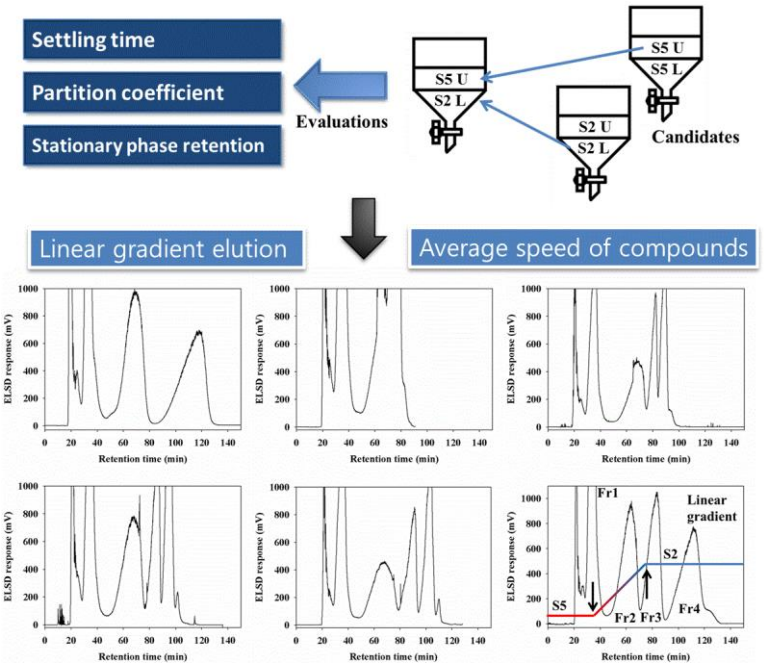

Figure 1. Schematic explanation of linear gradient countercurrent separation.

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APPLICATION OF COUNTER-CURRENT CHROMATOGRAPHY AS A POWERFUL FRACTIONATION TOOL. CASE STUDY: OBTAINING GRAM-SCALE SESQUITERPENOIDS FROM *TUSSILAGO FARFARA*

1024 PO Session

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Keywords: Gram-scale fractionation, Tussilago farfara*, Sesquiterpenoids*

Natural extracts from plants, source of biologically active metabolites, are exploited for drug or health supplement developments. However, its chemical complexity and diversity requires time-consuming multi-step purification procedures. *Tussilago farfara*, one of the medicinal plants of the family Asteraceae, contains diverse sesquiterpenoids, which show pharmacological activities. Minor sesquiterpenoids with similar chemical properties make pure compound isolation difficult in one-step CCC separation. Also, low contents of the sesquiterpenoids in raw material require efficient fractionation procedure.
In this study, new approach for enriching sesquiterpenoids from the buds of *Tussilago farfara* was developed. CCC operation was performed by HSCCC (TBE-1000A, Tauto Corp., China: coil volume: 1000 mL; 3.0 mm tube i.d.). 1.8 g of sesquiterpenoids-enriched fraction was obtained from 66.8 g crude extract of *Tussilago farfara* in a single CCC operation, with separation time of 7.5 hrs. For enriching target mixture, only 0.95 L water and 2.95 L organic solvents in total was used including extraction. Also, quantification studies of three major sesquiterpenoids in each fraction of different fractionation methods were conducted. CCC operation results shows the best efficiency compared to the conventional multi-step fractionations processed in series: solvent partition and open column chromatography. Considering its lab scale CCC device, solvent consumption, and processing time, this method enables powerful product recovery with high quality enrichment.

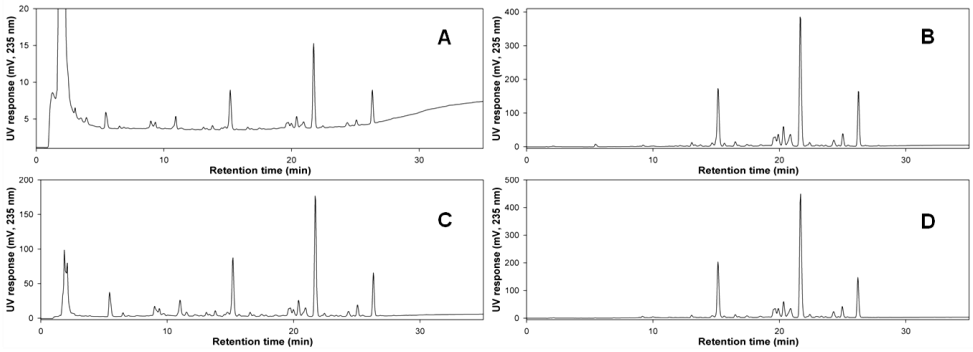

Figure 1. HPLC-UV chromatogram. (A) Crude extract; (B) CCC, (C) *n*-hexane, and (D) 100 % methanol fraction.

Table 1. Efficiency of three fractionation methods in recovery and solvent consumption.

Extract (66800 mg) from 200 g of raw material; 3.3, 6.7 and 2.5 mg/g of compounds 1, 2, and 3, resp.					
Fractionation tool		**CCC**	**Solvent partition (n-hexane)**	**Open column (Diaion HP-20)**	
				90 % MeOH	**100 % MeOH**
Yield (mg)		1784	3827	220	930
Compound 1 (mg/g), (% recovery)		119.3 (96.7)	55.2 (96.0)	54.7 (5.5)	125.3 (53.0)
Compound 2 (mg/g), (% recovery)		248.9 (99.0)	106.1 (90.5)	103.7 (5.1)	272.2 (56.4)
Compound 3 (mg/g), (% recovery)		93.8 (98.8)	35.1 (79.3)	34.3 (4.5)	82.2 (45.1)
Solvent consumption (mL) (including extraction)	**Aqueous**	950	2300	3500	
	Organic	2950	2300	5100	

PREPARATIVE SEPARATION OF EUPHORBIA FACTORS FROM *EUPHORBIA LATHYRIS* BY COUNTERCURRENT CHROMATOGRAPHY

1025 PO Session

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Keywords: Euphorbia *factors,* Euphorbia lathyris*, One-step separation*

This work demonstrates preparative one-step separation of *Euphorbia* factors from the seeds of *Euphorbia lathyris* using countercurrent chromatography with low cost. CCC operation was performed by HSCCC (TBE-1000A, Tauto Corp., China: coil volume: 1000 mL; 3.0 mm tube i.d.). Four major euphorbia factors: euphorbia factor L8 (19.6 mg), euphorbia factor L1 (128.8 mg), euphorbia factor L2 (56.2 mg), and euphorbia factor L3 (118.9 mg) were obtained from 8.8 g of the crude extract of *Euphorbia lathyris* in a single CCC operation. Including the extraction process, only 0.5 L water and 1.9 L organic solvents in total was used to isolate the target compounds. Isolated compounds were above 95 % in purity, as determined by HPLC (280 nm) and LC-MS analysis. This is the first report that euphorbia factors from the seeds of *Euphorbia lathyris* were successfully separated by CCC.

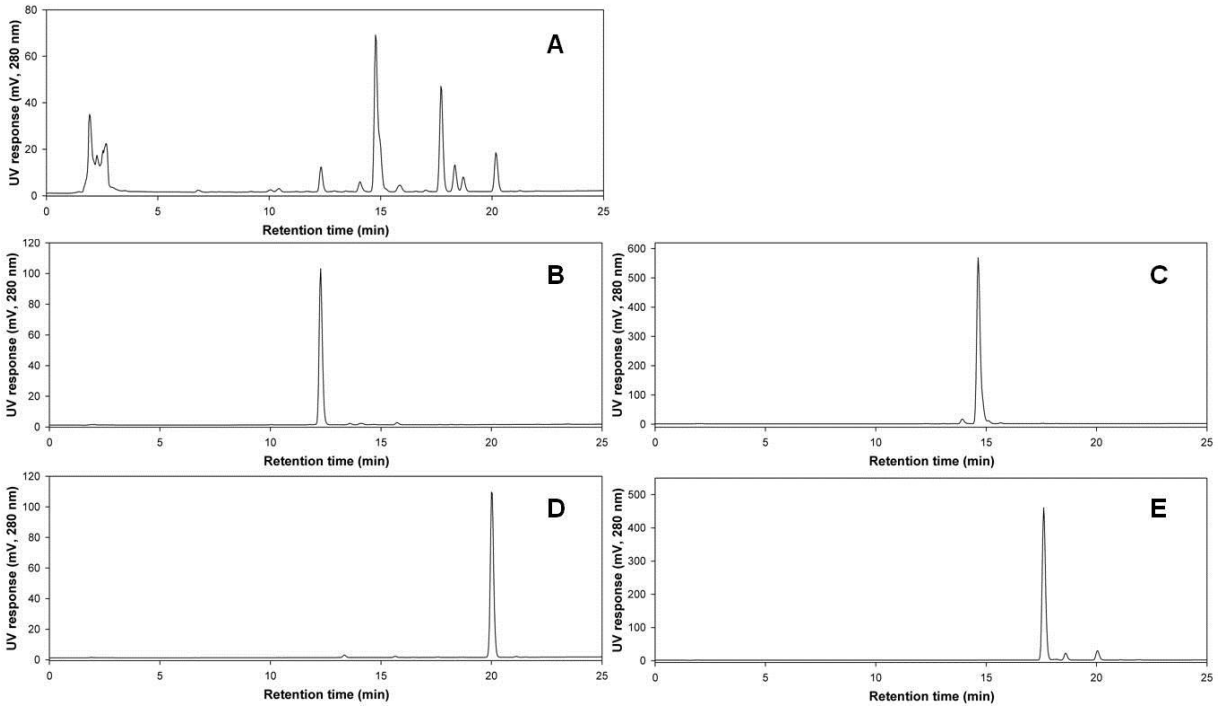

Figure 1. HPLC-UV chromatogram. (A) Crude extract; (B) Euphorbia factor L8; (C) Euphorbia factor L1; (D) Euphorbia factor L2; (E) Euphorbia factor L3

A NEW PROCESS FOR THE ANALYSIS OF MASTIC GUM AND ISOLATION OF BIOACTIVE TRITERPENS AND POLYMER

1026
FP
Session VII

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Keywords: mastic gum, pH zone step gradient, ^{13}C NMR dereplication, supercritical fluid chromatography

Mastic gum is a natural resin obtained from *Pistacia lentiscus* L. var Chia after "hurting" the trunk and branches. This high-value product is collected for more than 2,500 years and used in traditional medicine for various gastrointestinal disorders as well as in perfumery, dentistry and as a spice and flavoring in Mediterranean cuisine (1). Chemically, mastic gum consists of acidic and neutral triterpenes (about 75%) and polymers (25%). Due to the high complexity of this material the purification of the bioactive compounds is time consuming and requires multi-stage separation procedures to usually recover small amounts of compounds [2]. The present work aims to develop a rapid and effective process for the isolation of the main mastic gum constituents. The first step consists in the fractionation of crude mastic gum extract by using a novel CCC method which combines in the same run the pH zone refining and step gradient elution modes. The experiment starts by treating the row material with the biphasic system *n*-hexane/EtOAc/EtOH/H_2O 8:2:5:5 (v/v/v/v) in pH zone refining mode in order to fractionate the acidic triterpenes. Thereafter, a step gradient elution mode takes place by passing the lower phases of the same biphasic system in ratios 8:2:7:3, 8:2:8:2 and 8:2:9:1 (v/v/v/v) in order to separate the neutral triterpenes. Finally the column is extruded with *n*-hexane, resulting in the recovery of pure polymers. The separation was performed in a 300 mL CPE column leading to the effective fractionation of 7 g of mastic gum in only 2 hours. The chemical composition of each CPE fraction was established by ^{13}C NMR dereplication and 2D NMR analyses. Further analysis of enriched CPE fractions by supercritical fluid chromatography SFC-CO_2 using a chiral column led to the purification of the main triterpenic isomers of the mastic gum. This process can be considered as a new approach for the isolation of bioactive compounds from mastic gum. Furthermore, the proposed CCC methodology could be also applied for the effective fractionation of numerous plant extracts containing complex mixtures of neutral and acidic or basic components.

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Acknowledgment

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DEVELOPMENT OF A TWO-DIMENSIONAL SEQUENTIAL CENTRIFUGAL PARTITION CHROMATOGRAPHY PROCESS FOR THE PREPARATIVE SEPARATION OF TERNARY MIXTURES

1027 PO Session

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Keywords: two-dimensional separation, ternary mixture, continuous separation, sequential centrifugal partition chromatography (sCPC)

Preparative liquid-liquid chromatographic processes often require the isolation of components of intermediate elution speed from complex mixtures. When performing batch injections, peak cutting is usually necessary to obtain pure fractions of these target components, resulting in decreased productivity and yield. “Trapping” an intermediate component inside the column while eluting the neighboring compounds during multiple cycles of ascending and descending steps (1,2) can be used to achieve higher system throughput. However, this operating mode cannot be run continuously, since the process must eventually be stopped for collection of the non-eluted component. Continuous processes are often preferred in industrial settings for their high productivities and ability to be highly automated. An option for continuous separation of ternary mixtures is the two-dimensional liquid-liquid chromatography process reported by Couillard et al. in 2005 (3). In the proposed set-up, two two-column units are connected in series. In the first unit, a ternary mixture (A, B, and C) is continuously injected and fractionated into two product streams eluted sequentially during multiple cycles of ascending and descending steps. One product stream contains one component (A), while the other contains two (B and C). The two-component product stream (B and C) is fed directly to the second unit. Two product streams are again obtained, this time containing one component each (B or C).

The aim of this study was to extend an existing model-based design approach for the separation of binary mixtures using sequential centrifugal partition chromatography (sCPC) (4) for selection of the operating parameters in two sCPC units for the two-dimensional separation of (pseudo-)ternary mixtures. Before developing the continuous separation process, each sCPC step was evaluated individually. A short-cut design method was used along with simulations based on the equilibrium cell model for selection of the operating parameters such as feed and mobile phase flow rate, duration of the ascending and descending steps, and feed concentration. Several options for integraton of the two sCPC steps for continuous operation were explored, and strategies for adapting the product stream from the first unit to the feed stream of the second unit were addressed. The parameter selection approach was validated experimentally for each of the sCPC steps using a model mixture of three components of similar molecular structure (ethyl paraben, propyl paraben, and butyl paraben).

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CONTINUOUS FRACTIONATION OF MULTICOMPONENT MIXTURES WITH SEQUENTIAL CENTRIFUGAL PARTITION CHROMATOGRAPHY

1028 PO Session

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Keywords: continuous separation, sequential centrifugal partition chromatography (sCPC), multicomponent mixture

Sequential centrifugal partition chromatography (sCPC) is a cyclic liquid-liquid chromatographic process in which the feed stream is continuously introduced between two columns connected in series. The products are collected sequentially at opposite ends of the two-column set-up during multiple cycles consisting of two elution steps (ascending and descending). This concept was first reported by Couillard et al. in 2005 (1). A model-based design approach for the selection of the biphasic system and unit operating parameters for the separation of two-component mixtures using sCPC was previously described (2,3). The objective of this work was to extend this design approach to continuous pseudo-binary separations of multicomponent mixtures. Additionally, a strategy for maximizing system throughput while maintaining stable, predictable operation was developed (4).

The model mixture used in this investigation consisted of four components with similar molecular structure: methyl paraben (A), ethyl paraben (B), propyl paraben (C), and butyl paraben (D). Three separation tasks were performed as proof-of-concept of pseudo-binary fractionation: production of pure A, production of pure D, and separation of A and B from C and D. The durations of the ascending and descending steps were selected under ideal conditions (no dispersion effects) using a short-cut design method (2) so as to obtain the desired pure product streams from the quaternary mixture. Simulations based on the equilibrium cell model were used to verify that high purities could be obtained with the selected unit operating parameters under non-ideal conditions as well. The three pseudo-binary fractionations were performed with a global feed concentration of 8 mg/mL. Purities over 99% were achieved in all experiments.

A strategy for increasing the system throughput was then explored. In sCPC, higher throughput can be attained by increasing the feed concentration and/or the feed flow rate. When determining the maximum feed concentration, it must be considered that high solute concentrations inside the column can lead to changes of the initial volume ratio and compositions of the phases of the biphasic system. Partition coefficients of the solutes may be affected as well. Under such conditions, stable and predictable operation becomes impossible. Therefore, physical properties and partition coefficient measurements were performed at varying global concentrations of the paraben mixture in the biphasic system. The linear ranges of the partition coefficients were limited at a global concentration of 20 mg/mL. This corresponded to the concentration above which changes in the phase volume ratio were observed. The maximum feed concentrations allowing for operation in the linear range of the partition isotherm were determined by simulations. For production of pure D, this was found to be a global feed concentration of 18 mg/mL. As predicted by simulations, experimental product purities of ≥ 99% were obtained for this separation. Throughput was three times greater and solvent consumption three times lower than in the corresponding proof-of-concept experiment. The results of this study show that after determining the linear ranges of the partition coefficients, the short-cut method can be used in combination with the equilibrium cell model for improvement of sCPC unit separation performance.

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QUICK SELECTION OF SOLVENT SYSTEM FOR COUNTER-CURRENT CHROMATOGRAPHY SEPARATION WITH ONE SIMPLE HPLC METHOD

1029
FP
Session VI

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Keywords: solvent systems, HPLC method, GUESSmix, relationship

Counter-current chromatography (CCC) is widely used in the separation and enrichment of bioactive agents from complex samples, such as Chinese herbal medicines, fermentation and synthetic crude (1-3). The choice of an appropriate solvent system for CCC is a critical step in the purification of complex samples. However, it is still very laborious to find a suitable solvent system. The classical approach is to choose solvent system by K values or directly with analytic CCC (4). Previously, the authors discovered that an initial relationship could be established between HPLC elution system and the HEMWat system for CCC. This study is taking much broader approach to investigate the relationship between the gradually increasing of MeOH in MeOH/H_2O elution system for HPLC and HEMWat system S-8~S+8 (5) with GUESSmix, a mixture of 21 commercially available natural products and based on that, a quick selection of CCC solvent system for complex sample separation could be obtained by HPLC chromatogram. Furthermore, three complex herbal medicine sample types were also tested in this work to prove the generality of the method.

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SCALE UP PURIFICATION OF MONOSACCHARIDES FROM CRUDE HYDROLYSED SUGAR BEET PULP

1032
FP
Session VIII

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Keywords: monosaccharaides, sugar beet pulp, biorefinery

The isolation of component sugars from biomass represents an important step in the bioprocessing of sustainable feedstocks such as sugar beet pulp. Centrifugal partition chromatography (CPC) is proposed to be used as an alternative to multiple resin chromatography steps to isolate component monosaccharides from a hydrolysed sugar beet pulp pectin fraction (1). An ethanol - ammonium sulphate (300 g/L) phase system (0.8:1.8 v/v) was used in ascending mode to fractionate three sugar fractions (L-rhamnose, L-arabinose and D-galactose, and D-galacturonic acid) from a synthetic crude in a single step. Sample was prepared in the stationary phase to prevent solubility issues but was not shown to have a detrimental effect on separation performance based on synthetic crude separations. The optimised conditions were then scaled up from 200ml Kromaton CPC instrument to 2L RotaChrom CPC unit. The latter has different cell design allowing to achieve much higher flow rates with no loss in separation performance. To match the *g*-field level the rotational speed was dropped to 450rpm; while the mobile phase flow rate was increased up to 150ml/min providing 70% of initial stationary phase retention. The separation time was shortened by 50% with better resolution between target peaks. The separation, therefore, improved and further increase in scale and throughput are possible.

The present study is a proof of concept and the throughput is below the maximum loading capacity of the 2L instrument used.

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SEPARATION OF 5,6-DIHYDRO-A-PYRONES FROM *HYPTIS MONTICOLA* BY HIGH-SPEED COUNTRCURRENT CHROMATOGRAPHY (HSCCC)

1033 PO Session

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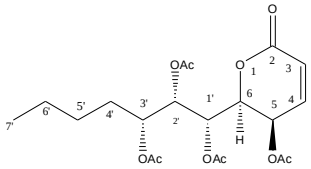
Keywords: Hyptis monticola*, Lamiaceae, alpha-pyrones, monticolides A-G*

Hyptis monticola Mart. ex Benth (Lamiaceae) is an endemic high-altitude grassland Brazilian species. Currently, about 580 species of *Hyptis* are known and the main phytochemicals described in the literature are terpenoids, alpha-pyrones, flavonoids and lignans. Alpha-pyrones are secondary metabolites endowed with cytotoxic properties towards a series of cancer cell lines [1]. To date, to the best of our knowledge, there is no record of the separation of this class of substances by countercurrent chromatography. Thus, the aim of this study was to develop a rapid method to isolate these compounds from the dichloromethane (DCM) extract from leaves of *H. monticola*. The plant was collected in Vale das Videiras, Rio de Janeiro, Brazil at 1.239m altitude. Leaves were dried, powdered and extracted with EtOH: H_2O (7:3). The resulting extract was subjected to liquid-liquid partitions with solvents of increasing polarity: hexane, CH_2Cl_2, EtOAc and *n*-BuOH. For the selection of the two-phase solvent system for HSCCC, eleven different ratios of the HEMWat solvent system (hexane: EtOAc: MeOH: H_2O) were tested due to the versatility of this quaternary system and because of its range of polarities. The following proportions were tested: **1** (3:1:1:0.5); **2** (3:2:1:0.5); **3** (2:3:1:0.5); **4** (1:1:1:1); **5** (2:1:1:1); **6** (3:1:1:1); **7** (1:2:1:1); **8** (0.8:1:0.8:1); **9** (1:0.8:1:0.8), **10** (0.5:1:0.5:1) and **11** (1:0,5:1:0.5). The results were visualized by TLC and solvent system **8** was chosen. The distribution coefficient (*k*) of the main compounds in the DCM extract were calculated by HPLC, from their peak areas in the chromatogram, and ranged from 0.6 to 10.8. The DCM extract was fractionated using the 95 mL coil of a Quattro HTPrep apparatus, with a flow rate of 3 mL/min (upper phase as mobile), 860 rpm. A total of 65 fractions (3 mL) were collected with the rotation on. Afterwards, 30 more fractions were collected pumping out the mobile phase. Fractions 14-21 afforded a tetracetylated 5,6-dihydro-alpha-pyrone which was named Monticolide A. Fractions 40-49 afforded a mixture of two triacetates named Monticolides B and C, which interconvert into each other by transesterification reactions. Fractions 56-67 afforded a mixture of Monticolide C and a diacetate, named Monticolide D. Frations 77-84 contained a mixture of three diacetates, with a free hydroxyl in C-5 of the lactone ring. They were named Monticolides E-G. Scale-up studies aimed at obtaining larger amounts of these compounds which allowed for the preparation of their Mosher esters and determination the absolute stereochemistry of Monticolides B-G.



Monticolide A

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COUNTERCURRENT CHROMATOGRAPHY WITH OFF-LINE DETECTION BY HPLC-ESI-MS/MS FOR THE SEPARATION AND IDENTIFICATION OF SAPONINS FROM *AMPELOZIZYPHUS AMAZONICUS*

1034 PO Session

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Keywords: Ampelozizyphus amazonicus, Rhamnaceae, triterpene saponins

Ampelozizyphus amazonicus Ducke (Rhamnaceae) is a climbing shrub native to the Amazonian region, where its bark and roots are used in the folk medicine to prepare a beverage to cure and prevent malaria, as well as a tonic and fortifier, among other uses (1). The main compounds cited in the literature are jujubogenin glycosides saponins (2,3,4). The crude extract of the saponins is too complex for any kind of structural identification and HPLC separation was not sufficient enough to resolve this issue. Therefore, the aim of this work was to obtain saponin concentrates from bark ethanol extract of *Ampelozizyphus amazonicus* by Countercurrent Chromatography (CCC) and to identify them by HPLC-HRMS and MS^n.

The bark ethanol extract was partitioned between water and in hexane, ethylacetate and butanol in the sequence. The butanol-rich phase was then fractionated by CCC with hexane - ethyl acetate - butanol - ethanol - water (1:6:1:1:6; v/v) solvent system yielding 5 group fractions. The first and third groups were further separated by CCC with dichloromethane - isopropanol - metanol - water (6:3:2:4; v/v) and ethylacetate - ethanol - water (1:0.2:1; v/v) solvent systems, respectively. The collected fractions from these two runs were analyzed for structural identification by HPLC-HRMS and MS^n. Group 1 contained mostly saponins with α- and β-amyrin skeletons. In Group 3 jujubogenin glycosides and keto-dammarane-type triterpene saponins with a C31 skeleton were the main compounds (Figure 1), indicanting that CCC was able to sort the saponins according to their skeletons. Thereby less complex samples could be analyzed by HPLC-ESI-MS/MS. Studies are in course to the complete elucidation of their structures.

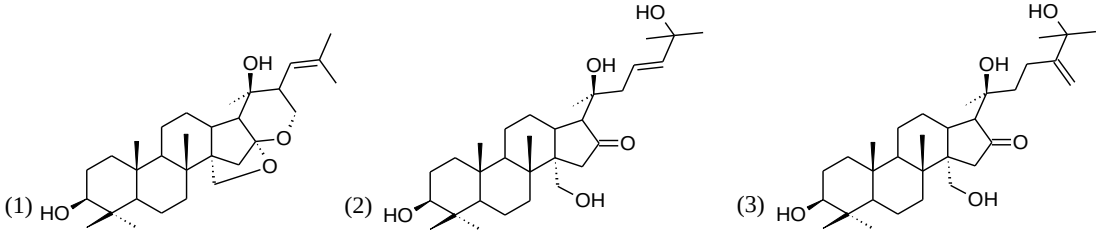

Figure 1. Saponin aglycone skeleton types present in group 3 fractions: (1) jujubogenin; (2;3) keto-dammarane.

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FAPERJ, CAPES, CNPq

ALKALOIDS FROM TRICLISIA DICTYOPHYLLA BY PH-ZONE REFINING CCC

1035 PO Session

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Keywords: Triclisia dictyophylla*, alkaloids, Plasmodium, pH zone refinement*

Triclisia dictyophylla Diels (Menispermaceae) is a climbing plant or scrambling shrub of the lowland dense rain-forest with stems that can be up to 30 m long and 10 cm in diameter. The plant occurs from Liberia to West Cameroon and in East Cameroon to Zaïre and Angola. The plant is harvested from the wild for local medicinal use [1]. A methanol extract of the stem bark of *T. dictyophylla* showed strong activity against *Plasmodium falciparum in vitro* and significant effect against *Trypanosoma brucei* [2]. Previous phytochemical studies on this plant revealed the presence of bisbenzylisoquinolines phaeanthine, *N*,*N*'-dimethyl phaeanthine, tetrandrine, trigilletine, cocsuline and trigilletimine from roots and stems. Oxo-isoquinoline *O*-methylmoschatoline and indenoisoquinoline triclisine were further obtained from stems. Analyses of the leaves showed bisbenzylisoquinolines stebisimine, obamegine, gilletine and isogilletine-*N*-oxide togheter with a morphinan alkaloid tridictyophylline [3-5].

The aim of this study was to develop rapid methodology for the isolation of tertiary and quaternary alkaloids from *T. dyctyophylla* by using pH zone refining CCC. A series of test tube partitioning tests were initially performed in order to select the best solvent system for the fractionation of the crude ethanol extract from stems of *T. dictyophylla* and then, the concentration of the retainer trimethylamine (TEA) and the eluter HCl were studied. The first solvent system family tested was HEMWat in various ratios but the results showed that a more polar solvent system was necessary. So, EBuWat was tested instead. A series of ratios (x:y:10) were investigated varying from x:y = 9:1 to 5:5. EBuWat 5:5:10 was chosen for optimizing the concentrations of TEA and HCl. After testing several concentrations of TEA in the upper organic phase and HCl in the lower aqueous mobile phase, 1g of the crude ethanol extract was injected in the 98mL coil of the Quattro HTPrep instrument, with the solvent system ethyl acetate-butanol-water 5:5:10, upper organic phase with 60mM TEA as stationary phase and aqueous lower phase with 5mM HCl as mobile phase, 2mL/min. Fractions of 4mL were collected. The present investigation led to the isolation of four major compounds from the stems of *T. dictyophylla*. Identification of the isolated compounds is being carried out by ^{1}H and ^{13}C NMR and by ESI-MS spectral data.

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OPTIMIZATION OF SAMPLE INJECTION IN COUNTER-CURRENT CHROMATOGRAPHY

1036
FP
Session XI

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Keywords: sample injection, GUESS mix, pharmaceuticals mix, sample loading, throughput

Counter-current chromatography (CCC) is well known for its high capacity of sample loading (1). However, it is still very much trial & error method when it comes to loading studies. The classical approach is to dissolve sample in one or both phases of two-phase solvent system and then inject sample solution without adjusting flow rate. Recently the authors developed a novel strategy, which allowed to increase loading by up to 40% by using “the best solvent” approach for preparing sample solution and by splitting sample injection into three stages. This strategy was well demonstrated on a separation of pre-purified natural product extract (2).

This study is taking much broader approach to investigate how solvents miscibility in the system and sample type affects the flexibility of the proposed injection strategy. Heptane-ethyl acetate-alcohol-water solvent systems, where alcohol is either methanol (HEMWat) or ethanol (HEEtWat), were compared as starting point. Methanol, as the aqueous phase modifier, is not miscible with heptane while ethanol is.ihua

Two sample types were tested in this work. One is mimicking a natural product extract – multicomponent mix within a wide range of polarity with an active compound (or two-three actives) at the content no more than 15%. The model sample was based on GUESS mixture of natural product molecules (3). Whereas the second model sample was mimicking a synthetic mix containing 2-5 components in a close polarity range with the main compound up to 70%. The latter sample was based on pharmaceutical molecules used by the authors in their previous study on method development (4).

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ADDITIVE MANUFACTURING: WHAT CAN IT DO FOR THE COUNTER-CURRENT CHROMATOGRAPHY RESEARCHER

1037
FP
Session X

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Keywords: Additive manufacturing, columns, rapid prototyping, 3D printing

Additive manufacturing (3D printers) is becoming widespread with sales of over 200,000 printers in 2015 and annual growth rates of over 20%. 3D printing is being widely used for making prototypes in various research fields (1), which reduces production time tremendously in comparison with using traditional engineering approaches. Thus, it would be advantageous to apply this new technology in the counter-current chromatography (CCC) research environment to rapidly produce different bobbin geometries and change column materials.

Therefore, a variety of components required for making a CCC column have been produced using a stereolithography (SLA) 3D printer (3Dsytems Inc. Viper SI2) from a photopolymer UV curing epoxy resin (Accura® Xtreme). This resin has similar properties to CNC machined polypropylene parts of CCC analytical columns at the Advanced Bioprocessing Centre.

Parts manufactured include snap-fit column spools and union fittings, splined interchangeable bobbins, flying lead moulds, threaded flying lead fittings, and counterweights for an analytical scale column. The advantages and disadvantages of the resin in rapid prototyping of parts for use in high cyclic *g*-fields will be discussed in detail. Advantages include reduced overall column weight, very short build times, and the ability to incorporate standard HPLC tubing fittings without modification due to snap fit plastic moulding. These parts have been tested on a standard analytical CCC one-bobbin instrument at the ABC labs and shown to be robust enough for testing of new column designs including standard sets of retention/resolution studies.

The potential to extend the design space into direct metal laser sintering, whereby highly robust lightweight metal parts can be produced, will also be discussed.

Acknowledgments

The authors would like to thank Krishna Burugapalli for assistance operating the SLA 3D printer.

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COMPUTATIONAL FLUID DYNAMICS MODELLING OF SECONDARY FLOW IN COUNTER-CURRENT CHROMATOGRAPHY INSTRUMENTS

1038
FP
Session XII

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Keywords: computational fluid dynamics (CFD), column shape, aspect ratio, secondary flow

Recent studies have used counter-current chromatography instruments for field-flow fractionation (FFF) in a cyclic *g*-field (1, 2), generally with a single liquid carrier and in a variety of column shapes. As a consequence of the curved tubing, a secondary flow is generated in the liquid carrier which enhances the mixing within the column.

To improve understanding of this phenomena and to enable its control depending on a tubing shape, computational fluid dynamics (CFD; Ansys CFX and Fluent software) was used to model the flow in rectangular columns with different aspect ratios (0.32 and 3.1). The secondary flows observed in these models were compared to those present in standard circular tubing of the same cross-sectional area. The secondary flow in case of the rectangular tubing being aligned horizontally to the *g*-field was seen to have similar velocities to that of the round tubing (0.157 m/s cf. 0.154 m/s), whereas the rectangular tubing aligned vertically to the *g*-field showed increased secondary flow velocities (0.20 m/s). Furthermore, the high velocity area in the vertically aligned tubing was far more uniform than in the other tubing shapes.

The simulation models were setup for standard fluids used in FFF experiments, including water and alcohols. The obtained results allow to draw some conclusions in regard to tubing selection for the fractionation process.

Analysis is at present being extending to include a range of other tubing shapes, and solvents.

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THE ROTIFY ® BENCH-TOP CENTRIFUGAL PRECIPITATION CHROMATOGRAPH

1040
FP
Session IX

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Keywords: Centrifugal precipitation chromatography, proteins, carbon nanotubes

A prototype of the centrifugal precipitation chromatograph has been fabricated and more recently modified to improve the flow tubing arrangement. The versatile protein fractionation device has been used and shown capabilities to separate proteins out of complex extracts, and perform affinity and immunochemistry procedures (1). A sample can be passed through the system simply to perform buffer exchange or dialysis in just 75 min. Two high density polyethylene plates clamp a dialysis membrane (6000-8000 MW cutoff) between a spiral flow channel that has continuous flow on the top and flow in reverse direction on the underside. The sample flow is through tubing that is clamped above the center of the rotor and passes through a side rod (black in photo) that also holds the outflow tubing and in-flow and out-flow of the gradient solution that passes on the other side of the membrane. In the photo, the tubing passes through gears underneath the rotor and up into the center of the rotor to connect with the spiral channels within the disk. When the system is centrifuged at 2000rpm, a protein mixture is passed in one channel at 50ul/min and a decreasing gradient of a precipitating agent is pumped at 5 or 1 ml/min entering at the opposite end of the channel. The small MW molecules pass through the membrane and in time the high MW molecules precipitate and move, re-dissolve and precipitate again at individual rates, such that they are separated and are eluted out the other end and collected. The process and structure of the device will be described. We have passed samples of carbon nanotubes and have removed other high MW constituents. The method should be useful also for purifying protein conjugates.

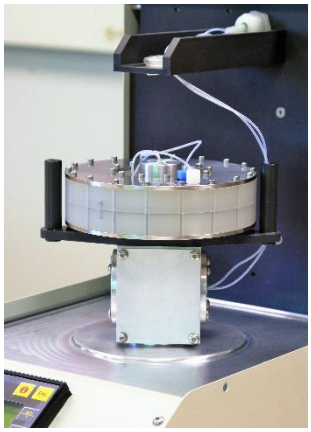

Figure 1. Close up of the Rotify®

Table 1. Gradient Solvent Systems

Analyte	Sample channel	Gradient channel
Proteins	Aq. buffers	95% sat. $AmSO_4$ – 0% Decreasing acetone concentration (2)
Carbon nanotubes	1% SDS	33% Am acetate – 0%

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CONCENTRIC COILS FOR COUNTER-CURRENT CHROMATOGRAPHY

1042
FP
Session X

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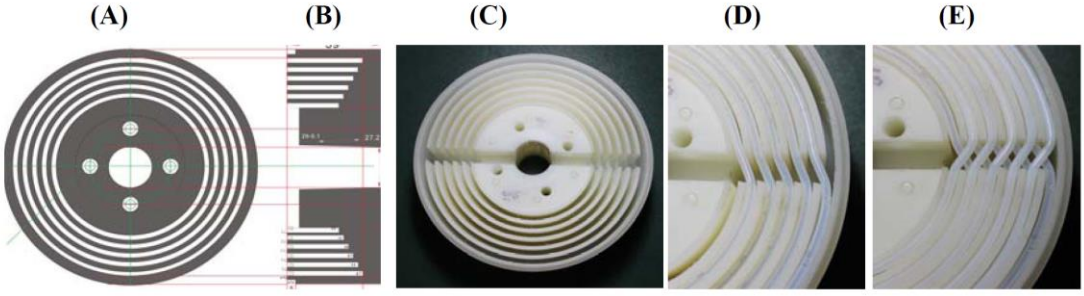
Keywords: Concentric coils; Helical coils; Spiral coils; Natural Products isolation; CCC column

Countercurrent chromatography (CCC) is an efficient separation technique in which two immiscible liquid phases are used and hold in the CCC column under high centrifugal force. Due to lack of the complications resulted from the support matrix, CCC has been widely used for the preparative purification of natural and synthetic products. Historically, modern CCC originated from the helical coil planet centrifuge in early 1970s by Yoichiro Ito [1, 2]. The helical coiled tube was found to be efficient to be used as a CCC column including type I and type J flow-through CPC, multi-layer CPC, eccentric multi-layer CPC, high-speed CCC, cross-axis CPC, low-speed CCC, and even high-performance CCC. Usually, helical coils can provide stable axial driving force in the centrifugal field but lack efficient pressure gradient of centrifugal force, especially in type-J high-speed CCC, which attain the unilateral hydrodynamic equilibrium in the coiled tube. Recently, spiral coils or disk assemblies [3, 4] were found to improve the retention of the stationary phase and enhance the partition efficiency for high-speed and low-speed CCC in the isolation of peptide and proteins. However, the spiral coils still lack the axial gradient of the centrifugal field for type J CCC, although it can attain an extra centrifugal gradient in the radial direction. More recently, we built conical coils to form centrifugal force gradients in both axial and radial directions [5]. Compared with helical and spiral coil CCC, conical coil CCC not only put the CCC column in a two-dimensional centrifugal field, but also provided a potential centrifugal force gradient both in axial and radial directions. The extra centrifugal gradient made mobile phase move faster and enabled higher retention of stationary phase and better resolution. However, above coiled columns were still difficult for fractionation and machine balance. Therefore, in this work, we developed a simple concentric coil or disk assembly as CCC column. This new assembly was shown to be efficient in the separation and purification of several natural products.



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STRATEGY FOR PH-DEPENDENT TAILING IN COUNTER-CURRENT CHROMATOGRAPHY: ALKALOIDS OF *NELUMBO NUCIFERA* GAERTN AS EXAMPLES

1043
FP
Session VIII

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Keywords: pH-dependent tailing, leading peak; alkaloids; liensinine; supermolecular separation

Counter-current chromatography (CCC), as a unique liquid-liquid partition chromatography, has been found to be a very efficient separation technique for resolving the complex natural products. Due to lack of support matrix, it eliminates some complications resulted from solid supports such as in irreversible solute adsorption, contamination, reaction and deactivation in the process of common chromatographic separation [1]. In addition, CCC may also eliminate tailing of solute peaks although the tailings of basic compounds in reversed-phase and normal liquid chromatography is a major problem that commonly occurs in the separations of pharmaceutical, peptides and proteins [2]. Usually, tailing has been linked with residual silanol groups on the surface of the solid supports such as silica stationary phase that remain after surface modification with reversed-phase groups. However, little is known about peak tailing or leading in CCC [3-5]. In this work, we found that in some cases, CCC can also produce significant tailing or leading peaks, as illustrated in the following figure. Therefore, this work aimed to developed several strategies to resolve the peak tailing in the separation of alkaloids of *Nelumbo nucifera* GAERTN.

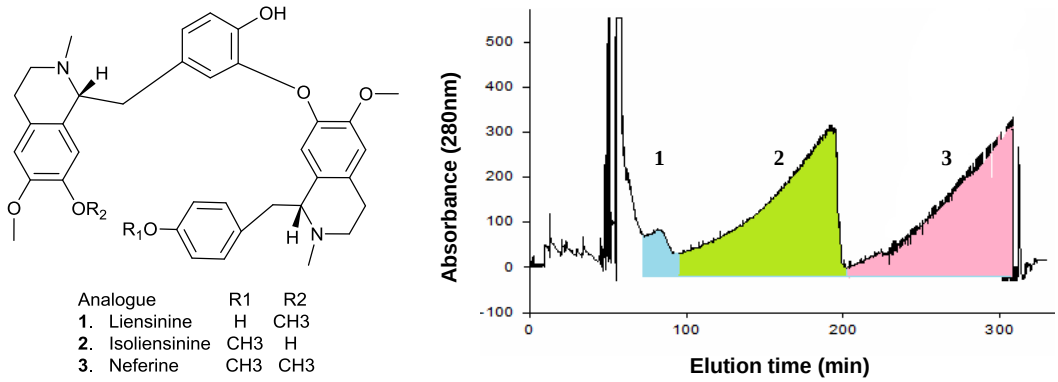

Figure 1. Leading peaks in the separation of alkaloids of *Nelumbo nucifera* GAERTN using lower phase of HEMWat systems as mobile phase.

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ROOM TEMPERATURE IONIC LIQUIDS-BASED SALTING-IN STRATEGY FOR COUNTER-CURRENT CHROMATOGRAPHY

1044 PO Session

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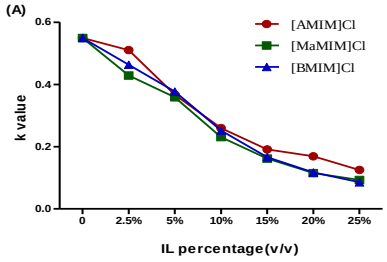
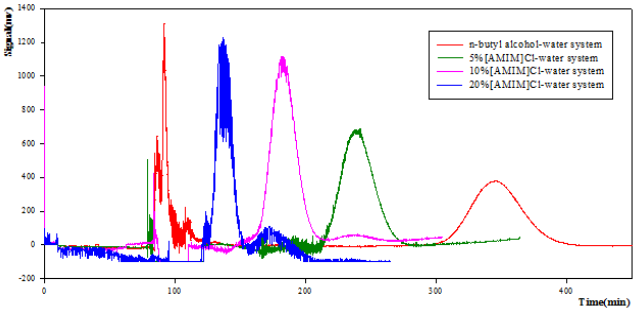
Keywords: Room temperature ionic liquids; Salting in; Salting out; Solvent system selection; Solvent systems.

Counter-current chromatography (CCC) is an unique liquid-liquid partition chromatography without complications caused by solid-support matrix and has been shown to be very efficient for the separation and purification of natural products. However, CCC separation is still a challenge. Usually, a common CCC separation process involves several steps such as the selection of solvent systems, instrumentation and separation theory mode. The selection of appropriate solvent system for target compound(s) is the first and the most important step in CCC separation, which probably accounts for 90% of the whole work [1]. Salting-out is a very common but not simple physical phenomenon extensively exploited by biopolymer science, ion-exchange chromatography, and counter-current separations. It has been proposed and used for the partition study of protein, amino acid, and hydrophilic natural products. Previous studies indicated salting-out was efficient method for separation of several natural products using some one-component inorganic/salt-containing systems such as NaCl, KCl, $(NH_4)_2SO_4$, KNO_3 as salting-out agents [2]. Different from the inorganic salts, we recently found that some organic salts such as sodium dodecyl sulfate (SDS) may play a “salting-in” role and make the partition coefficients of the solutes decrease with the increase of its concentrations [3]. Room temperature ionic liquids (RTILs) are well known organic salts being made up of cations and anions. Unlike classical viscous organic liquids, they are actually molten salts with melting point close or below room temperature. Their liquid state allows them as both the mobile and stationary phases in CCC [4]. Recent study [5] showed a similar salting-in properties with SDS. In this work, we report several RTILs-based salting-in strategy for CCC based isolation of natural products.





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NOVEL NON-AQUEOUS BIPHASIC SOLVENT SYSTEMS IN CENTRIFUGAL PARTITION CHROMATOGRAPHY

1045 PO Session

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Keywords: deep eutectic solvents, non-aqueous biphasic systems, hydrophobic compounds

In Centrifugal Partition Chromatography and Countercurrent Chromatography, the most frequently used biphasic solvent systems are composed of three to four solvents of different polarity, such as heptane, hexane, ethyl acetate, methanol, ethanol and water. Even though these systems cover a wide range of polarities, they are often unsuitable for the separation of highly hydrophobic and hydrophilic compounds. The partition coefficients are very high or very low for such compounds because they preferentially dissolve in one of the two phases. For the purification of active compounds from plant extracts and biotechnological products, there is a strong need for new biphasic solvent systems especially for the separation of hydrophobic mixtures (1).

Deep Eutectic Solvents consist of two (or three) compounds capable of associating with each other to form a eutectic mixture with a melting point substantially lower than that of each individual compound (2).

In this work, the applicability of Deep Eutectic Solvents (DES) as solvents in liquid-liquid chromatography was evaluated. For this purpose, the partition coefficients of several natural compounds of different hydrophobicity in heptane/ethanol/DES biphasic systems were determined. Since many DES are hygroscopic, the influence of the DES composition and the presence of water in the biphasic system on the partition coefficient was also examined. In addition, several process-relevant biphasic system physical properties, such as the density and viscosity of the phases, were evaluated (3).

Finally, the suitability of DESs as solvents in liquid-liquid chromatography was demonstrated at pilot scale using a Centrifugal Partition Extractor column (SCPE-250-BIO from Armen Instrument, France).

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K-TARGETED PURIFICATION OF C-GLYCOSYLFLAVONES FROM *VITEX AGNUS-CASTUS* BY ORTHOGONAL COUNTERCURRENT METHODS

1046
FP
Session X

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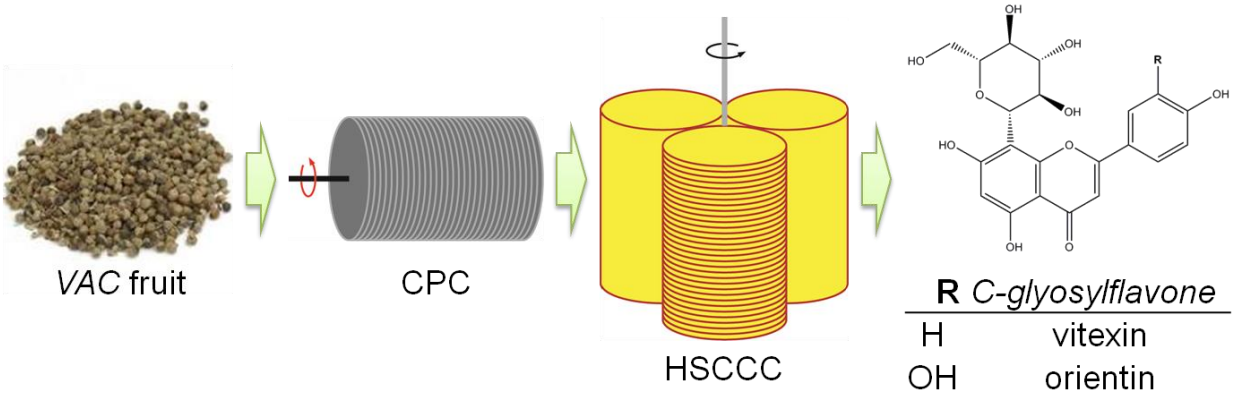
Keywords: CPC, HSCCC, LC-MS, shake flask, vitexin, orientin

C-glycosylated flavones, including orientin, isoorientin, vitexin and isovitexin, are minor but biologically significant constituents of chaste-tree (*Vitex agnus-castus* L.) fruits, which are used as a botanical supplement to treat PMS and postmenopausal symptoms. The partition coefficient, or *K*-value, is the ratio of the concentration of a compound in each phase of a biphasic solvent mixture and is a physicochemical property of a particular compound in a particular solvent system. This value can be used to predict retention volume (V_{ret}) in a countercurrent separation (CCS) procedure. The *K*-values of *C*-glycosylflavones present in complex botanical fractions have been determined in a number of solvent system families (*ter*AcWat, EEtWat, ChMWat) using the shake-flask technique. Relative LC-MS quantification allowed for the determination of *K*-values of multiple compounds of interest from complex extracts and CS fractions. *K*-values of *C*-glycosylflavones were also compared to interfering compounds, such as *O*-glycosylflavones, to optimize separations to avoid co-elution of unwanted compounds. This *K*-value library was used to develop targeted centrifugal partition chromatography (CPC) and high-speed countercurrent chromatography (HSCCC) methods to purify *C*-glycosylflavones.



AN INTEGRATED PROCESS FOR THE RECOVERY OF HIGH ADDED-VAUE COMPOUNDS FROM EXTRA VIRGIN OLIVE OIL USING SOLID-SUPPORT FREE LIQUID-LIQUID EXTRACTION AND CHROMATOGRAPHY TECHNIQUES

1047 PO Session

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Keywords: Extra Virgin Olive Oil, phenols extraction, step-gradient elution, oleocanthal, hydroxytyrosol.

Extra virgin olive oil (EVOO) is recognized as the main factor responsible for the health and nutritional benefits of the Mediterranean diet. This ancient oil is an abundant source of phenolic compounds with high health-benefiting properties (1, 2). The major phenolic compounds identified in olive oil belong to three different classes: simple phenols, secoiridoids and lignans. Hydroxytyrosol, oleocanthal and oleaceine are three olive oil phenolic compounds of particular interest due to their important biological properties (3, 4). In the present study an integrated extraction and purification process for a direct and productive recovery of high added value compounds from EVOO is purposed by using liquid-liquid extraction/chromatography techniques. Two different extraction methods were studied and developed on a laboratory-scale Centrifugal Partition Extractor (FCPE300®). The semi-continues multi-dual mode method consisting of several “extraction-recovery” cycles and the co-current elution method for the continuous recovery of the phenolic fraction. Food grade n-hexane was used to dilute olive oil in proportion of 3:2 v/v (feed mobile phase) and mixture of ethanol/water 3:2 v/v was used to extract the phenolic fraction. In total, 20 L of EVOO were treated resulting in the recovery of 26 g of total phenolic fraction. The obtained phenolic mixture was then fractionated using preparative-scale Centrifugal Partition Chromatography (FCPC1000®) combined to a sequential step-gradient elution procedure. The biphasic solvent systems were composed of n-hexane, ethyl acetate, ethanol and water in different volume proportions (X/Y/2/3, v/v/v/v) producing organic mobile phases with increasing polarities. In a single run of 4 hours, 910 mg of oleocanthal, 882 mg of oleacein and 104 mg of hydroxytyrosol were successfully produced from 5 g of phenolic extract with mean purities of 85%, 92%, and 89% respectively. Additionally, 791 mg of MFOA and 421 mg of elenolic acid were directly obtained from the same fractionation also in acceptable purity (>85%). The structure elucidation of the isolated compounds was achieved by NMR analysis. The proposed combination of the CPE/CPC techniques for the production of high value bioactive compounds from olive oil is described for the first time and represents a short process with high productivity. The above methodology could be also applied for the effective treatment of numerous edible oils and the recovery of their bioactive constitutes.

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Acknowledgment

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ISOLATION OF METABOLITES FROM MANGROVE PLANT *RHIZOPHORA MANGLE* BY COUNTERCURRENT CHROMATOGRAPHY

1048 PO Session

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Keywords: Rhizophora mangle, diterpenes, HSCCC, antibacterial

Mangrove plants are potential sources of biologically active compounds with numerous traditional and medicinal uses[1]. Brazil's coastline is 7408 km, from which 6786 km contain mangrove forests, covering 25.000 km^2. *Rhizophora mangle* (Rhiphoraceae), known as red mangrove, is a Brazilian native tree and occurs in all mangrove areas[2]. The plant is commonly used for the extraction of tannins (15-36% of the dry bark)[3] but phytochemical studies on the species also reported the isolation of flavonoids and triterpenes from the leaves[4].

In this work, three diterpenes – manool, jhanol and steviol – and a benzaldehyde – *p*-oxy-2-ethylhexyl benzaldehyde (**Figure 1**), were isolated from the hexane extract of aerial roots by countercurrent chromatography (Quattro HT-Prep, 98mL Vc) using a biphasic non-aqueous solvent system composed of hexane-acetonitrile-methanol 1:1:0.5 (v/v/v). The fractions and isolated compounds from *R. mangle* are being screened for their minimum inhibitory concentration (MIC) following CLSI guidelines for *Cryptococcus neoformans* T1-444, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538 and *Escherichia coli*. The literature reports the presence of kaurane, labdane, pimarane and beyerane diterpenes besides several aromatic compounds in Rhizophoraceae[4]. However, as far as we know, only steviol was previously isolated from this plant family.

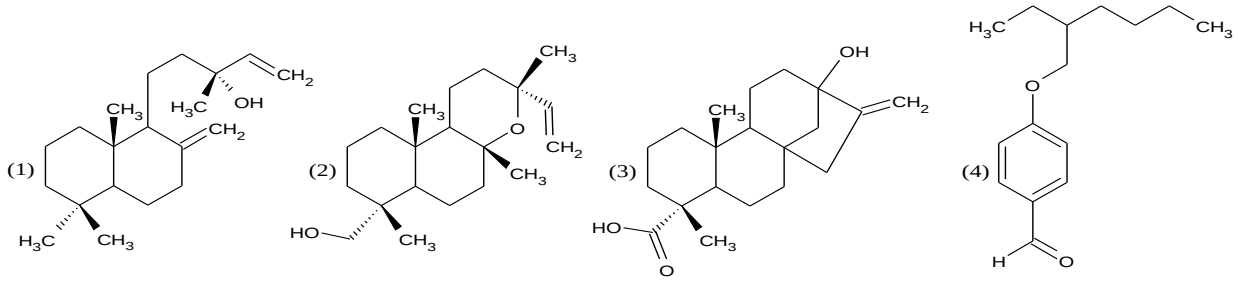

Figure 1. Isolated compounds (1) manool, (2) jhanol, (3) steviol and (4) p-oxy-2-ethylhexyl benzaldehyde

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MULTIPLE DUAL-MODE CPC AS AN EFFICIENT TOOL FOR THE PURIFICATION OF CAULERPENYNE FROM *CAULERPA TAXIFOLIA*

1049 PO Session

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Keywords: Caulerpa taxifolia*, caulerpenyne, centrifugal partition chromatography, isolation, purification.*

Caulerpenyne (Cyn) is a cytotoxic compound first isolated in 1978 from *Caulerpa prolifera*.(1) This molecule, constituted by a diacetoxybutadiene moiety (Fig. 1), exhibited a wide range of biological properties such as, antibacterial properties (2) and antitumor activities by inhibiting the growth of several human cancer cell lines (3).

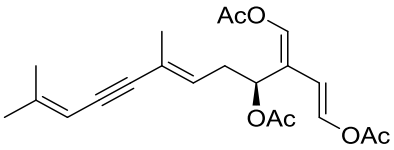

Figure 1. Caulerpenyne

Several industrial applications can be found for Cyn, so there is a need to produce and isolate it in high quantities. Since Cyn purification is time- and solvent-consuming, it is crucial to find a more green process to obtain pure Cyn with lower costs.Among the current chromatographic techniques, Centrifugal Partition Chromatography (CPC) seemed more appropriate to our objectives. Indeed, it allows low solvent consumption, can be used from analytical to preparative scale and is less time-consuming than other techniques. In the literature, CPC has been used to fractionate and/ or isolate bioactive compounds and has shown its efficiency in the purification process of natural products from diverse species.

Thus, in our study, we used CPC to separate and purify the metabolites within the crude extract of *Caulerpa taxifolia* algae. Multi dual-mode was thus chosen with the Arizona N solvent system (Hexane-Ethyl acetate--Water-Methanol 1:1:1:1) to afford Cyn with high purity (more than 98%). Further scale-up assay was made without loss of purity. Direct application of the method on the *C. taxifolia* crude extract gave caulerpenyne with 0.2% yield (*i.e.* 5 times more than with classical chromatographic techniques) and more than 98% purity. The structure of Cyn was confirmed by ^{1}H NMR and compared with previously published data (4).

Acknowledgements: E. Sfecci is the recipient of a thesis grant from the "Conseil Régional Provence Alpes Côte d'azur". M. Mehiri research is supported by the french program ENVI-Med "MEDIBIO", the ANR/Investissements d'Avenir program via the OCEANOMICs project (grant #ANR-11-BTBR-0008), and the H2020 European program via the EMBRIC project.

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CAULERPENYNE FROM *CAULERPA TAXIFOLIA*: A COMPARATIVE STUDY BETWEEN CPC AND CLASSICAL CHROMATOGRAPHIC TECHNIQUES

1050 PO Session

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Keywords: Caulerpa taxifolia*, caulerpenyne, centrifugal partition chromatography, purification*

Caulerpenyne (Cyn) (**1**) is a cytotoxic compound first isolated in 1978 from *Caulerpa prolifera*.(1) This molecule, constituted by a diacetoxybutadiene moiety, exhibited a wide range of biological properties with mainly antibacterial properties (2) and antitumor activities by inhibiting the growth of several human cancer cell lines.(3) Several industrial applications are possible for Cyn, so there is a need to produce and isolate it in large quantities. Since Cyn purification is time- and solvent-consuming, it is crucial to find a more green process to obtain pure Cyn with lower costs.

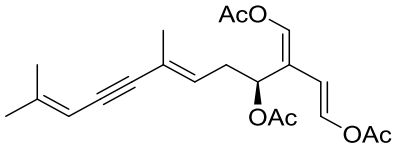

Figure 1. Caulerpenyne

Thus, in our study, Cyn has been purified from *C. taxifolia* crude extract with two different techniques: Centrifugal Partition Chromatography (CPC) and classical chromatographic techniques. CPC method involves only the CPC step with 0.2% yield (dry weight). On the other hand, other chromatographic techniques traditionally used imply at least three steps: (i) a liquid-liquid extraction, (ii) a size exclusion chromatography, and finally (iii) a diol column chromatography with a 0.04% yield (dry weight). Among the current chromatographic techniques, CPC seemed the more appropriate to our objectives for several reasons: (i) it allows low solvent consumption, (ii) it can be used from analytical to preparative scale and (iii) it is less time-consuming than other techniques. In the literature, CPC has been used to fractionate and/or isolate bioactive compounds and has shown its efficiency in the purification process of natural products from diverse species/ origin. The comparative study showed CPC to be faster at lower costs for Cyn isolation, and increased the extraction yield significantly.

Acknowledgements: E. Sfecci is the recipient of a thesis grant from the "Conseil Régional Provence Alpes Côte d'azur". M. Mehiri research is supported by the french program ENVI-Med "MEDIBIO", the ANR/Investissements d'Avenir program via the OCEANOMICs project (grant #ANR-11-BTBR-0008), and the H2020 European program via the EMBRIC project.

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RATIONAL DEVELOPMENT OF CONICAL COLUMNS ON J-TYPE COUNTER-CURRENT CHROMATOGRAPHY FOR PROTEIN SEPARATION USING AQUEOUS-TWO PHASE SYSTEMS

1051
FP
Session IX

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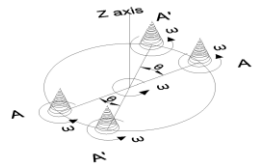
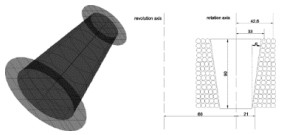
Keywords: Conical column, aqueous two-phase system, physical model, protein separation, HSCCC

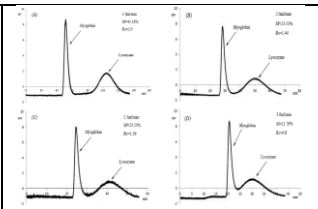
Purification and separation of large biomolecules and nanoparticles using counter-current chromatography (CCC) has been challenging yet much desired. Such usage invariably resorts to aqueous two-phase systems. For handling large molecules like proteins, it is notoriously difficult to achieve both sound stationary phase retention and phase mixing. With J-type CCC, successes have been shown for 2D spiral columns[1] and 3D toroidal columns[2]. This work considered both the pros and cons of 2D spiral columns and 3D cylindrical spiral columns, and then developed conical column geometry for protein separation and isolation (Fig. A)[3].



A: the J-type planetary motion of conical coil column. θ:elapsed rotation angle of the rotor; ω:rotation speed



B: Preparation of the the new conical bobbin (left) and winding details (right) of the column.



C: Separation of myoglobin and lysozyme using an ATPS. Phase system: 14% w/w PEG1000 and 12.5% K_3PO4, 1000 rpm rotation.

Each location is quantified by angular value φ

$$x = R\cos\theta + l\varphi\sin\alpha\cos(2\theta + \varphi x)$$
$$y = R\sin\theta + l\varphi\sin\alpha\sin(2\theta + \varphi)$$
$$z = l\varphi\cos\alpha$$

D: Mathematic description for the trajectory of arbitrary point on conical coil column on J-type CCC (R: revolution radius; *l* : positive scaling factor; φ : angular position; α : taper angle)

The conical column can be formed by lifting the center of the 2D spiral column in the vertical direction perpendicular to the 2D spiral plane. When the radius gradient over the horizontal orientation gradually reduces to complete disappearance, the conical column will then be converted to a 3D spirally wound cylindrical column. The factors influencing the stationary phase retention and phase mixing include β value, rotation speed (ω), winding pattern [Fig.B (right)], conical taper angle (α), angular position (φ). When β>0.5, stationary phase retention is favored but not for phase mixing. On the contrary, when β<0.5 (particularly β<0.25), phase mixing is favored, but not for phase retention[4]. Consequently, stationary phase retention always trades off with phase mixing for such a system. The taper angle should be between 5-15º following a compromise between stationary phase retention and phase mixing. The column winding pattern [Fig B(right)] ensures that better mixing takes place at those locations suitable for more column volume. We will show that a conical column strikes a balance for having the advantage of a spiral column for improved stationary phase retention and at the same time for having the advantage of a 3D spiral column for improved phase mixing. Based on our virtual CCC results, we then built a conical column in a real physical form (Fig. B). With the use of an ATPS, model proteins lysozyme and myoglobin have been well resolved (Fig. C).

In conclusion, this work illustrates an example for constructing and then analyzing the physical model (the conical column in this case) as a virtual column (Fig. D). Experiment vindicated the efficacy of the conical coil column for protein separation.

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COMBINING SEVERAL ELUTION MODES TO SEPARATE COMPOUNDS FROM COMPLEX MATRIX

1052 PO Session

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Keywords: countercurrent chromatography; elution mode; complex matrix

Counter-current chromatography (CCC) is a unique chromatographic separation technology which based on the liquid-liquid partition and extraction without the solid support stationary phase. It has been widely used for the preparative and analytical separation, isolation and purification of various products due to its advantages in separation. However, simultaneously separate multiple compounds, especially in large scope of polarity range has been an intractable problem in CCC application. Usually, it is necessary to use several different solvent systems and multiple separations to isolate many compounds from a complex matrix because of the low number of theoretical plates of CCC. This leads to the great increase of solvent consumption and extend the time of the separation.

In such a case, we consider combining several elution modes to achieve separation of compounds with a large polarity scope from complex matrix (such as extract of traditional Chinese medicine) in a single CCC separation. In this separation, a high-speed countercurrent chromatography using the gradient elution combined elution-extrusion mode to separate compounds in the extract of *Cynomorium*. It is first and important step to select a suitable two-phase solvent system in a CCC separation (1). A classical solvent system consist of *n*-hexane-ethyl acetate–methanol–water (HEMWat) was used to achieve this separation. A series of HEMWat biphasic systems with different composition were screened to optimize the solvent system for CCC separation and their partition coefficients (K values) of the compounds were measured. Two solvent systems were selected (HEMWat 5 and 6). Firstly the lower phase of HEMWat 6 was used as stationary phase and the upper phase as mobile phase. After the hydrodynamic equilibrium was established throughout the column, the sample solution was injected. Then the upper phase was continuously pumped in the column and eluted. After eluted with a column volume of upper phase of HEMWat 6, the mobile phase changed from the upper phase to HEMWat 5 and kept on eluting. Then, continued eluting a column volume with the upper phase of HEMWat 5 as mobile phase. Next began the extrusion process and the lower phase of HEMWat 5 was introduced into the column to push out the stationary phase in the column. The fraction was collected with an automatic fraction collector.

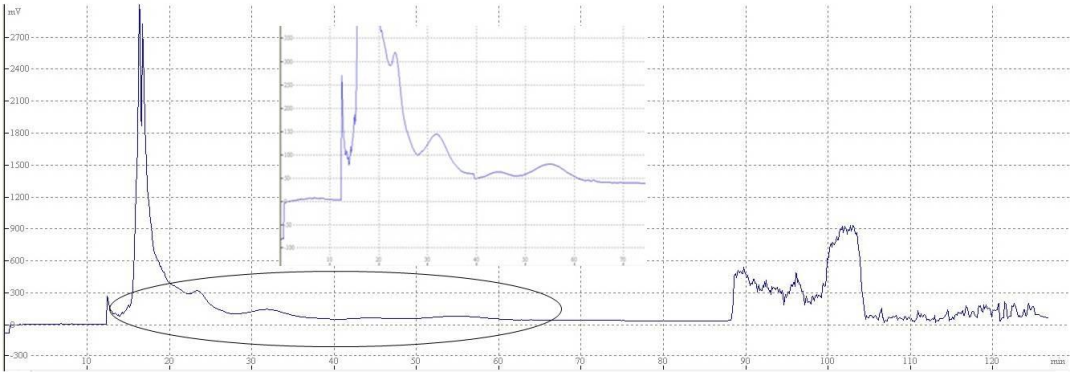

Figure 1. HSCCC chromatogram of combined elution modes separation

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SEPARATION AND PURIFICATION OF ACTIVE COMPONENTS FROM LYCIUM BARBARUM L. BY HSCCC USING DUAL-MODE ELUTION

1053 PO Session

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Keywords: Lycium barbarum *L., dual mode, antioxidant*

Lycium barbarum L. is a Solanaceous defoliated shrub that is widely grown in arid and semi-arid regions of Northwestern China, Southeastern Europe, and the Mediterranean areas. The fruits of *L. barbarum*, also called goji berry or wolfberry, have been used as traditional Chinese herbal medicine and functional food for more than 2500 years. *L. barbarum* has the functions of nourishing kidney and liver, improving fertility in men, and brightening eyes. In this work, a dual-mode elution was used in the separation of *L. barbarum* L. extracts by HSCCC with a two-phase solvent system composed of *n*-butanol–ethyl acetate–water (1:4:5). The separation was initiated by filling the multilayer coil column with the upper phase of the solvent system as the stationary phase. The lower phase was then pumped into the column using the head-to-tail mode for elution. After a run of a certain time, the inlet and outlet of the column were switched, and the upper phase, which was originally used as the stationary phase, was eluted in the tail-to-head direction through the column. Three compounds with a wide range of polarity were separated using this method. They are 5-hydroxymethyl furfural, rutin and quercetin. The HSCCC chromatogram is shown in Figure 1. The antioxidant activities of the compounds were evaluated by the methods of DPPH radical scavenging assay. Rutin and quercetin showed high radical scavenging activities with the EC_{50} values being 20.07 ± 0.10 and 3.11 ± 0.03 μg/mL, respectively.

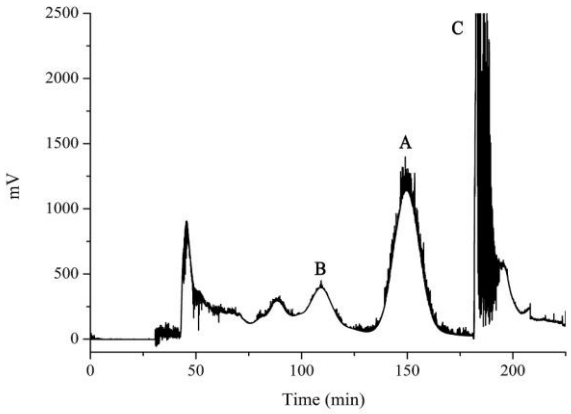

Figure 1. HSCCC chromatogram of the extract of *Lycium barbarum* L using dual-mode elution. The solvent system is *n*-butanol–ethyl acetate–water (1:4:5)

THREE SOLVENT SYSTEM CCC COMBINED THE USE OF O-CARBOXYMETHYL CHITOSAN AS AN ADDITIVE FOR SEPARATION OF CHEMICAL COMPONENTS IN *LYCIUM BARBARUM* L.

1054
FP
Session VI

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Keywords: three solvent system, O-carboxymethyl chitosan, solvent addictive, Lycium barbarum L .

Lycium barbarum (goji berry) is one of the most important traditional Chinese medicine. Their fruits are reported to have various biological activities and health-promoting properties. Therefore, the fruits and leaves have been used widely as vegetable medicines and functional tea in China, Southeast Asia, Europe, and North America (1). An increasing number of researchers have focused on the study of the active components, trying many different separation methods to obtain them. However, these methods exhibit some shortcomings such as a tedious process, time and labor consumption, solvent residue, low efficiency and so on. Recently, several applications of three-phase solvent systems in HSCCC separation have been reported. And this kind of HSCCC technique has potential application in separation of physiologically active constituents with a wide range of polarity in natural products (2,3).

A new three-phase solvent system was efficiently applied for high-speed counter-current chromatography to separate the secondary metabolites with a wide range of hydrophobicity in *L. barbarum* .The three-phase solvent system of n-hexane/methyl acetate/acetonitrile/water (4:3:4:3.5, v/v/v/v) was selected for high-speed countercurrent chromatography separation. The two phases (middle phase and lower phase) of a three-phase solvent system was as a stationary phase followed by elution with upper phase to separate the hydrophobic compounds. Then the mobile phase was switched to the middle phase to elute the moderately hydrophobic compounds, and finally the polar compounds were eluted out of the column with the lower phase. Although we get six peaks through the three-phase solvent system CCC separation, the resolution is not good enough. In order to improve the resolution, we will use the O-carboxymethyl chitosan (O-CMC) as a solvent additive. In Liu's research it was indicated that O-CMC could improve resolution not by increasing the retention of the stationary phase but by introducing intermolecular forces: hydrogen bonding interaction and electrostatic interaction (4). We will also investigated the effect of O-CMC concentration on *K* and *Rs* to optimize the CCC separation condition. Through this method, we expect to get a comprehensive separation of a wide variety of the complex sample in a one-step operation in a relative less time without any pretreatment.

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SEPARATION OF SAPONINS FROM *SILENE COLORATA* BY USING CENTRIFUGAL PARTITION CHROMATOGRAPHY

1055 PO Session

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Keywords: Silene colorata*, Saponins, CPC*

Saponins are a class of chemical compounds found in particular abundance in various plant species. There is a constant demand for these compounds for their amphiphilic properties that makes them natural foaming agents, especially for the cosmetic market, while they are also being promoted commercially as dietary supplements and nutraceuticals. The genus *Silene* (Caryophyllaceae) is known to contain triterpenoid saponins [1] and phytoecdysteroids [2]. *Silene colorata* is distributed over the Mediterranean basin, but little is known concerning the phytochemistry of the species. Previous work has focused on the identification of the main secondary metabolites in various *Silene* extracts, among which *S. colorata.* Especially, the content in saponins/sapogenins has been specified. There have been identified both two main sapogenin (aglycone moiety) types, triterpenes and steroids/phytoecdysteroids [3]. The co-existence of saponins and their aglycone forms in the extracts of *S. colorata*, as well as the fact that the polarity of these groups of compounds varies widely, makes it difficult to separate them and, moreover, quantify them. Several works exist focusing on analytical methods for the detection and quantification of saponins and/or sapogenins, individually or as a group of compounds [4]. Nevertheless, little work has been published regarding the separation of saponins in preparative scale. Especially from *S. colorata*, there exists no such work.

Centrifugal partition chromatography (CPC) is a preparative technology that proves to be very efficient for the separation of natural compounds. It has been successfully implied in the case of a hydroalcoholic *Silene colorata* extract that was further enriched by adsorption resin treatment, by optimizing the biphasic solvent system that effectively separates the saponins. Thus, 10 biphasic solvent systems were tested and compared between them and finally a system composed of n-Hex/EtOAc/BuOH/EtOH/H_2O at a volume ratio of 5:5:5:6:15 was found to be the optimal for the saponins separation. This was used in ascending mode, inside a 50ml rotor. A spectrophotometric method has been used for the determination of the total sapogenin content (TSC) for each extract and fraction of the CPC. The recovery of fractions that contain total saponins >95% has been achieved.

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DEVELOPMENT OF A COUNTER-CURRENT CHROMATOGRAPHY-BASED EXTRACTION METHOD FOR EMERGING CONTAMINANTS FROM RIVER WATER

1056
FP
Session VI

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Keywords: emerging contaminants, extraction, column material

Complex mixtures of emerging pollutants which include pesticides, biocides, personal care products, and pharmaceuticals are constantly present in the hydrosphere and may pose a risk to aquatic ecosystems and human health. This is becoming a major problem with ever growing density of population. The vast and ever-increasing number of chemicals, often present at very low (sub ng/L) concentrations, complicates their monitoring and subsequent regulation (1). In this context, counter-current chromatography (CCC), all liquid technique, was considered as a possible alternative to solid phase/membrane extraction as CCC has been used as a concentration method for trace level compounds before (2). The research explores the use of CCC as an extraction method for large volumes of water. In this mode, the mobile phase is the water sample potentially containing the pollutants, while the stationary phase is an appropriate organic solvent(s) with added extracting reagents to enhance the partitioning of target molecules. The model water samples contained a number of compounds, including pesticides, pharmaceuticals, and corrosion inhibitors, covering a wide polarity range with log P between 1 and 5. The preliminary results demonstrated that there is clear evidence of retaining certain types of molecules on the inner walls of a CCC column, possibly caused by adsorption. This led to further consideration of testing different types of column materials. A range of recovery efficiencies for the majority of these compounds and method development will be presented and discussed.

Acknowledgments

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COUNTERCURRENT CHROMATOGRAPHY FRACTIONS OF PLANT EXTRACTS WITH ANTI-TUBERCULOSIS ACTIVITY

1057 PO Session

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Key Words: plant extracts, anti-tuberculosis activity

The southwestern part of the United States has a large variety of indigenous plants, many of which have not been investigated for their medicinal potential, and only very few of which have had their extracts separated into the individual compounds they may contain. Samples of numerous plant species were received from that area from Richard Spjut, who is very highly knowledgeable of the various plant species of that region, including which species might be more likely to have medicinal properties than others. All were extracted with typical solvents, giving crude residues, some of which were subjected to countercurrent chromatography (CCC) separation.

Some of the crude residues and some of the CCC fractions were tested for anti-tuberculosis activity using both MABA and/or LORA methods. Activity was found in some samples in each of the two categories. Test results will be given, including comparisons of the anti-TB activity of certain CCC fractions, with the anti-TB activity of the crude residues from which they came, respectively.

ISOLATION AND PURIFICATION OF α-MANGOSTIN FROM INDONESIAN *GARCINIA MANGOSTANA* L. RINDS USING HIGH PERFORMANCE COUNTER-CURRENT CHROMATOGRAPHY

1058
PO
Session

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Keywords: α-mangostin, Mangosteen, Garcinia mangostana

Independence of drug raw materials has been a concern by the government of Indonesia lately and exploration of natural materials is expected to be the best solution, especially the exploration of various medicinal plants that exist in Indonesia. Mangosteen rinds with a content of xanthones and derivatives including α-mangostin is one of the medicinal plants that could potentially be developed as a source of raw material for medicine because it has antioxidant activity, anti-bacterial, anti-fungal, and anti-tumor. Laboratory testing so far indicates that α-mangostin is effective against several types of cancer cells, including breast cancer, liver, and leukemia. A research on isolation and purification of α-mangostin from mangosteen rinds had been done using Mini High Performance Counter Current Chromatography (HPCCC). This study aimed to obtain α-mangostin with high purity which can be accepted not only as drug raw materials but also as reference materials. The research was begun with preparation of the extract by maceration of the rind powder in 80% ethanol, then selection of a solvent system for HPCCC by testing the solubility of α-mangostin in the upper phase and the lower phase in HEMWat solvent (hexane, ethyl acetate, methanol, water) in various proportions. Sample injection was repeated 5 times without the top up of the stationary phase. The results showed that the best solvent system for the isolation of α-mangostin by HPCCC was HEMWat (5: 5: 10: 4 v / v) with K_D = 1, upper phase was used as the stationary phase while lower phase was used as a mobile phase. Samples were dissolved in the mobile phase before injection. Running time required for each injection was 25 minutes and the α-mangostin retention time ± 20 minutes. Total α-mangostin obtained was 38.0 mg with a purity of 98.13 ± 1.11% and 98.6% recovery Identification and α-mangostin assay were done by HPLC while characterization of α-mangostin isolated was conducted by NMR. In conclusion, this HPCCC had very efficient performance because the isolation and purification was done in one step with 5 times simultaneous injections. The purity of α-mangostin isolated was > 98% which can be accepted as drug raw material or as reference material.

Key words: α-mangostin, drug raw or reference material.

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PHYTOCHEMICAL INVESTIGATION OF ROOT OF ECLIPTA ALBA FOR ANTI-*MYCOBACTERIUM TUBERCULOSIS* CONSTITUENTS

1059 PO Session

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Keywords: Eclipta alba, Mycobacterium tuberculosis, Tuberculosis,

Tuberculosis, a global health security threat due to its contagious nature, is among the deadliest diseases of the current era killing almost 1 person in every three minutes. According to the WHO Global Tuberculosis report 2015, tuberculosis accounts for 30,000 deaths per week [1].Traditional medicines possess the great potential to act as rich source of therapeutic agents which can be of much importance to pharmaceutical industry.Climate, geography and soil of Pakistan are very suitable for the growth of different kinds of medicinal plants [2]. *Eclipta alba* (L) Hassk (syn: *Eclipta prostrata* Linn.Family: Asteraceae) is a commonly growing annual herb in moist soils of tropical Asia [3] .The use of this plant in traditional Chinese medicine is thousands of years old where it is mentioned variously as Eclipta herba or as “Mo-Han-Lian” [4]. In Ayurveda, it is reported as beneficial in case of bronchitis and asthma; stem has been used for treating tuberculosis and amoebiasis besides asthma. Accumulated evidences from reported literature point towards the existence of possible potential agents to combat TB.

This study was aimed towards the validation of reported traditional use of this plant and investigation into the possible active constituents through FCPC. The crude extract of the roots of *Eclipta alba* was first fractionated through vacuum liquid chromatography (VLC), and the resulting 41 fractions were combined (after matching of TLC bands) into 11 fractions. The most active fraction, ER-3, showed a dose dependent growth inhibitory effect against *Mycobacterium tuberculosis* (exhibiting 96%, 88% and 45% inhibition at 128, 64, and 32 μg/ml, respectively), which was subjected to FCPC. The solvent system used was hexane/TBME/Acetonitrile (9:2:10). The upper organic phase was used as mobile phase. While docosane was detected by GCMS as the main component of this fraction, it does not explain the bioactivity. While this indicates that minor constituents are reponsible for the observed *M.tb*. growth inhibitory effect, the data indicate that phytomedical rationale exists for the traditional use of *Eclipta alba* as an anti-TB remedy.

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ONLINE ENRICHMENT AND SEPARATION OF FIVE FLAVONOIDS COMPOUNDS FROM *MIKANIA MICRANTHA* USING MAGNETIC NANOMATERIALS COUPLED WITH HIGH SPEED COUNTERCURRENT CHROMATOGRAPHY

2002
FP
Session III

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Keywords: Flavonoids, Magnetic Nanomaterials, Countercurrent Chromatography

Mikania micrantha is an invasive weed. Flavonoids generally exist in weeds. Owing to its ability of antioxygenation, improving circulation, and cholesterol-lowering effects, the separation and enrichment of flavonoid components in weeds is important. In this study, we compared the adsorption capacity of six different magnetic nanoparticles to bind to flavonoid standards, then selected Fe_3O_4@SiO_2@DIH@EMIML magnetic nanoparticles to fill online polytetrafluoroethylene pipeline. A device of magnetic nanoparticles (MNPs) online pipeline combined with high speed countercurrent chromatography (HSCCC) through a six-way valve has been used to achieve online separation and enrichment of the five components simultaneously. Ethyl acetate-methanol-water (10:1:10, v/v) or *n*-hexane-ethyl acetate-methanol-water (1:1:1:1, v/v) was used for on-line separation of the astragalin, quercetin, luteolin, baicalein, and kaempferol. Five flavonoids targets have been enriched and separated. The method for separating flavonoids by on-line combination of magnetic nanomaterials and high speed countercurrent chromatography was established for the first time.

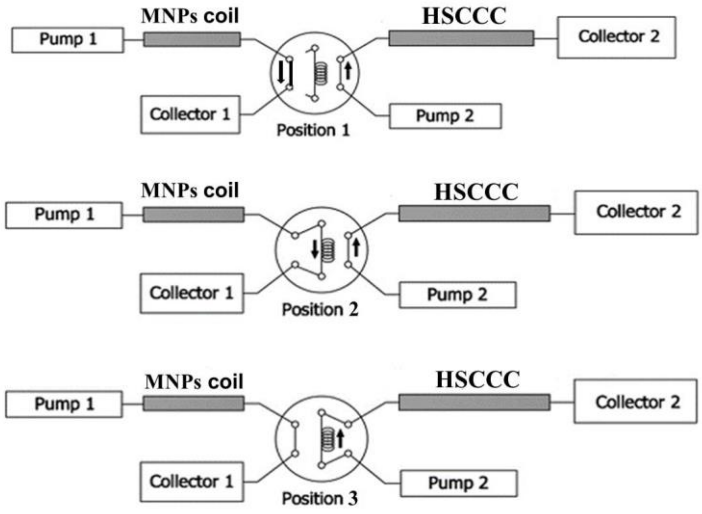

Figure 1. Scheme of the magnetic nanoparticles- high speed countercurrent chromatography system

ENANTIOSEPARATION OF AROMATIC ACIDS BY PRECOLUMN DERIVATIZATION COUNTERCURRENT CHROMATOGRAPHY

2011
FP
Session II

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Keywords: Aromatic acids; Countercurrent chromatography; Enantioseparation; Precolumn derivatization

The enantiomeric separation and analysis of chiral drugs has become essential since enantiomers exhibit different biological behaviour and may exert different pharmacodymic, pharmacokinetic, and toxicologic activities. Chromatographic enantioseparations could be divided into three categories: direct separation on a chiral stationary phase, enantioseparation by chiral mobile phase additive, and separation of diastereoisomer formed by precolumn derivatization with a chiral derivatization reagent. These methods have been substantially explored and used in traditional liquid chromatography and gas chromatography. In recent years, countercurrent chromatography has become increasingly attractive for enantioseparations due to its preparative capacity, and more than ten kinds of chiral selectors has been tested and investigated [1]. However, literature about enantioseparation by precolumn derivatization countercurrent chromatography, as far as we know, have not been published.

Enantioseparation of aromatic acids by countercurrent chromatograpy, including mandelic acid derivatives and 2-arylpropionic acid derivatives, have been mainly investigated in the recent years in our lab using different chiral selectors, such as β-cyclodextrin derivatives and L-proline derivatives [2-4]. It was found that some racemates couldn't be enantioseparated. Herein we want to report our recent study on the enantioseparation of some racemic aromatic acids by precolumn derivatization countercurrent chromatography, which was not separated in our previous work. (-)-Menthol was selected as the chiral derivatization reagent for esterification of racemic aromatic acids. A suitable biphasic solvent system was selected for separation of diastereoisomeric analytes, as shown in Figure 1.

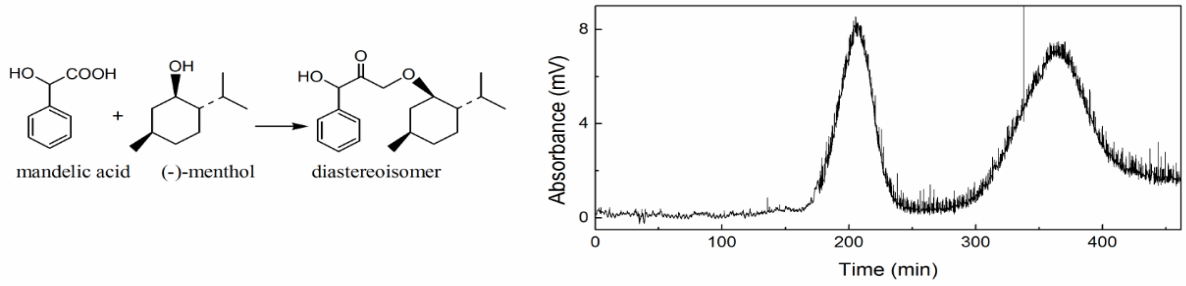

Figure 1. Esterification of mandelic acid with (-)-menthol and chromatogram of separation of diastereoisomer by countercurrent chromatography

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RAPID PURIFICATION AND SCALE-UP SEPARATION OF THREE MAKAMIDES FROM *LEPIDIUM MEYENII* USING HIGH-CAPACITY HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

2013
FP
Session I

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Keywords: Lepidium meyenii*; high-speed counter-current chromatography; makamides*

A prototype of engineering high-speed counter-current chromatograph (HSCCC) was designed containing two separation columns. Each of them is composed of three single-channel units. A rapid separation and scale-up approach has been developed by using this prototype to isolate the macamides, *N*-benzyl-(9*Z*,12*Z*)-octadecadienamide, *N*-benzyl-9*Z*-octadecenamide, and *N*-benzyl-9*Z*- octadecenamide, from a crude extract that was obtained from *Lepidium meyenii* by Supercritical Fluid Extraction (SFE). 1g of crude macamide extract was separated and purified by HSCCC with a two-phase solvent system composed of n-hexane-ethylacetate-methanol-water (7:2:5:1, v/v/v/v) with one unit in one step for about 360 min, and the fractions were analyzed by high-performance liquid chromatography (HPLC). This large scale preparative single step run yielded 226 mg *N*-benzyl-(9*Z*,12*Z*)-octadecadienamide with a purity of 97.0%, 313 mg *N*-benzyl-9*Z*-octadecenamide with a purity of 98.8%, and 152 mg *N*-benzyl-hexadecanamide with a purity of 95.4%. This is the first time that high-speed counter-current chromatography has been used to purify makamides in a multiple gram scale in less than 6 h and at such high purity of the final products.

SEPARATION AND IDENTIFICATION OF A NOVEL SUBSIDIARY COLOR OF THE COLOR ADDITIVE FD&C RED NO. 40 (ALLURA RED AC) USING SPIRAL HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

2017 PO Session

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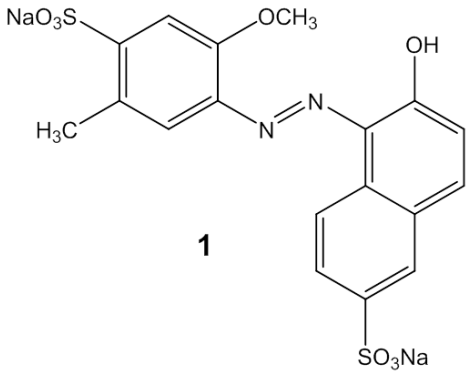
Keywords: affinity-ligand pH-zone-refining CCC; azo color additive

FD&C Red No. 40 (R40, Allura Red AC, Colour Index No. 16035) is a color additive permitted in the United States for coloring foods, drugs, and cosmetics. It consists mainly of the disodium salt of 6-hydroxy-5-[(2-methoxy-5-methyl-4-sulfophenyl)azo]-2-naphthalenesulfonic acid, **1**. During its manufacture, a host of synthetic by-products are produced, and they are found in various amounts in the final product. Before it may be used as a color additive, R40 is subject to batch certification by the U.S. Food and Drug Administration (FDA) to ensure compliance with limits on levels of impurities specified in the Code of Federal Regulations (CFR).

In the current study, a method based on spiral high-speed counter-current chromatography using the affinity-ligand pH-zone-refining mode was developed for the preparative separation of a subsidiary color impurity from a batch of R40. The impurity is often observed in HPLC chromatograms of batches of R40 submitted for certification, and its identity has not heretofore been known. Very few published works describe the separation of components of R40, and none involves use of preparative methods for separating subsidiary color impurities. For identification purposes in the present study, it was necessary to isolate the impurity, which represents only about ~0.6% of the dye. The chemical structure of the isolated impurity was then determined by NMR and LC-MS/MS.



1

COUNTERCURRENT SEPARATION OF NATURAL PRODUCTS: VERBENONE FROM ROSEMARY ESSENTIAL OIL

2025
FP
Session VI

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Keywords: Rosemary essential oil, Rosmarinus officinalis*, verbenone*

Countercurrent chromatography has become increasingly popular in the separation of natural products [1] due to its low solute decomposition, no irreversible solute adsorption (total recovery of injected sample) and high loading capability. Besides this, its relatively simple retention mechanism (liquid-liquid partitioning) allows working with many sample types. All this turns CCC into a robust technique for the preparative isolation of target compounds from plant extracts. Nevertheless, there are relatively few publications describing its use for the isolation of compounds of interest from essential oils.

Verbenone is an unsaturated bicyclic terpene ketone that possesses an odor reminiscent of camphor, menthol and celery, and has been actively pursued as a potential candidate for the protection of individual trees and forest stands because it decreases the response to pheromone in many bark beetle species. It can be naturally found in rosemary (*Rosmarinus officinalis* L.), Spanish verbena (*Verbena triphylla*), Spanish *Eucalyptus globulus* essential oils and other plants [2].

The aim of this work was to use countercurrent chromatography for the isolation of verbenone from rosemary essential oil. The essential oil was obtained from fresh leaves of rosemary, upon hydrodistillation in a Clevenger-type apparatus. Analyzes of the essential oil were performed by GC-MS and GC-FID systems. The identification of the components of the essential oil was carried out by comparison of mass spectral data with NIST 14 and Willey 275 libraries and also, by the calculated and experimental linear retention indices comparison. The major compounds identified were camphor (28.4 %), 1,8-cineole (15.0 %) and α-pinene (12.5 %). Verbenone (5.5 %) was a minor compound.

To make possible the separation of verbenone, it is important to choose an adequate solvent system. Due to the chemical composition of the essential oil, rich in hydrocarbon and oxygenated terpenes, five different solvent systems with low to medium polarities were chosen for test tube partitioning test selection: **A.** hexane-ACN 1:1, **B**. hexane-ACN-MeOH 1:1:0.1, **C**. hexane-ACN-EtOAc 1:1:0.1, **D**.hexane-EtOH-H_2O 4:3:1 and **E**. hexane-EtOH-H_2O 4:2:2 . Solvent systems **B**, **C** and **D** gave very similar results and hexane:EtOH:H_2O 4:3:1 was chosen for the purification of 113.7 mg of rosemary essential oil, as it doesn't contain ACN and so is considered more eco-friendly and less toxic solvent system. The purification was done using the 26 mL coil of an HTPrep apparatus, with a flow rate of 1 mL/min (upper phase as mobile), rotation speed of 860 rpm. The retention of stationary phase was 71 %. A total of 25 fractions (2 mL) were collected and verbenone was isolated from fractions 10-13. These fractions were analyzed by GC-FID and GC-MS and verbenone was identified, with a relative area of 100 %. So, CCC showed to be a capable tool to isolate verbenone, one of the minor constituents of rosemary essential oil.

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THE WORKING MECHANISM OF TOROIDAL COLUMNS ON J-TYPE COUNTER-CURRENT CHROMATOGRAPHS

2038
FP
Session X

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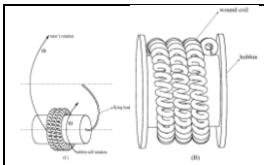
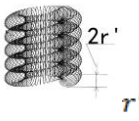
Keywords: Counter-current chromatography, toroidal column, mathematical model, protein separation and purification, optimization of toroidal column design

INTRODUCTION: To address the ever increasing challenges in biopharmaceutical industry, toroidal columns mounted on a J-type counter-current chromatograph (CCC) have applications potential[1,2]. Presently, the largest toroidal column bore size is 5mm and the largest processing capacity allows for 20 ml/min as mobile phase flow rate[1]. Compared to the most popular 3D cylindrical columns, how do we understand the reported encouraging protein separation outcomes and what can be done further to rationally design and improve this type of column?

PHYSICAL MODEL:

 **R:** rotor rotation radius **r:** bobbin self-rotation radius	 r': pre-tightly wound chain radius. An increase of 2π for φ value leads to one rotation yet moves the coil forward.	The toroidal coil augular location φ (an independent variable) is expressed by Cartesian coordinate system as, $x = R\cos\theta + \beta R\cos(2\theta + n\varphi) + r'\cos\varphi\cos(2\theta + n\varphi)$ $y = R\sin\theta + \beta R\sin(2\theta + n\varphi) + r'\cos\varphi\sin(2\theta + n\varphi)$ $z = \frac{r'n}{\pi}\varphi + r'\sin\varphi$ where *n* is the number of toroidal chains (with radius r') over a bobbin turn, and is determined by the r' value, and θ is the elapsed rotation angle of the rotor.
A: real appearance of the column in the CCC.	B: modelled appearance	C: Mathematic description for each location on a toroidal column on J-type CCC.

RESULTS & DISCUSSION: The J-type CCC planetary rotation creates a unique pattern of centrifugal force in terms of the rotary roration (quantified by the angle θ) and the column position (quantified by the locational angle φ). Two coordinate systems were used to study various physical interactions between the two liquid phases and the column internal wall surface. For the first coordinate system, we separated this CCC centrifugal forces into three perpendicular forces, namely the tangential (a_t), the normal (a_n), and the binormal force (a_b). For the second coordinate system, we separated the CCC centrifugal forces into two perpendicular forces: the tangential direction of the coil (a_t) and the plane orthogonal to the tangential direction. On the latter plane, the orthagonal force ($a_{\vec{N}+\vec{B}}$) is specified by its magnitude ($|a_{\vec{N}+\vec{B}}|$) and direction (by angle α).

Using the second coordinate system, it could now be observed that the combined normal force ($a_{\vec{N}+\vec{B}}$) rotates once for every 2π increment of the φ value for the toroidal column (i.e., for each pre-wound toroidal chain). In contrast, this pattern of the combined normal force ($a_{\vec{N}+\vec{B}}$) rotation takes place for each turn of column on the bobbin for the most popular 3D cylindrical spiral column[3,4]. In view of the fact that the toroidal column studied has ca. 20 toroidal chains for each turn on the bobbin, this means that for each bobbin turn of the toroidal column, the liquid inside has gone through ca. 20 times of pulsified mixing. With the column length being constant, it is observed that when the combined normal force ($a_{\vec{N}+\vec{B}}$) rotates once for the 3D cylindrical spiral column, this force has rotated ca. three times for the toroidal column. Such frequent change in direction of this normal force undermines establishment of the friction forces between the two liquid phases and also between the liquid phases and the column internal wall, and hence may well significantly reduces forces directly related to stationary phase retention. These results provide insights into both the mixing nature and the stationary phase retention mechanism, and formed a basis for the subsequent improvement of toroidal columns.

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