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Rotation planar extraction and rotation planar chromatography of oak (*Quercus robur* L.) bark

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Abstract

The versatile novel instrument for rotation planar extraction and rotation planar chromatography was exploited for the investigation of oak bark (*Quercus robur* L.). The same instrument enabled extraction of the bark, analytical proof of (+)-catechin directly in the crude extract and also its fractionation. Additionally, epimeric flavan-3-ols, (+)-catechin and (−)-epicatechin were separated by analytical ultra-micro rotation planar chromatography on cellulose plates with pure water as developing solvent. A comparison of the extraction of oak bark with 80% aqueous methanol by rotation planar extraction and medium pressure solid–liquid extraction was carried out and both techniques were shown to be suitable for the efficient extraction of oak bark. The raw extracts and fractions on thin-layer chromatography showed many compounds that possessed antioxidant activity after spraying with 1,1-diphenyl-2-picrylhydrazyl. Rotation planar fractionation of 840 mg of crude oak bark extract on silica gel gave 6.7 mg of pure (+)-catechin in one run.
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1. Introduction

Medium-pressure solid–liquid extraction (MPSLE) introduced by Nyiredy et al. [1] is an extraction technique based on the principles of diffusion-dissolving processes of parametric pumping which is well characterized [1,2]. A change in the intensive parameter of the system, e.g., temperature, pressure, pH or electric field, results in a reversible differential alteration of the distribution of components between the solid and the liquid phase. MPSLE can be performed in a medium-pressure liquid chromatography (MPLC) column. The same principal factors as in column chromatography, e.g., the geometry of the column, physico–chemical properties of the solvent, flow-rate and amount of solvent, pressure, equilibrium time, particle size, compactness and amount of material to be extracted, are valid in MPSLE [1].

Rotation planar extraction (RPE) is a forced-flow

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solid–liquid extraction (FFSLE) technique, where the extraction solvent(s) migrate(s) mainly through the action of centrifugal force [3,4]. A novel multifunctional separation instrument prototype ExtraChrom enables the rotation planar extraction of complex matrices since a planar column can easily be attached to it and then filled with the material to be extracted. Factors affecting the RPE process are basically the same as in MPSLE with the exception that the solvent is being driven by centrifugal force instead of a pump and the geometry of the column differs from MPLC columns used in MPSLE.

Rotation planar chromatography (RPC) is a forced-flow technique to be applied both for preparative, using the planar column, or analytical work. Four commercially produced RPC instruments are available on the market: Hitachi CLC-5 (Hitachi, Tokyo, Japan), Chromatotron (Harrison Research, Palo Alto, CA, USA), Rotachrom P (Petazon, Zug, Switzerland), Cyclograph (Analtech, Newark, DE, USA). However, only the ExtraChrom instrument enables the extraction, isolation and analysis of compounds from complex matrices [4]. This instrument consists of a separate extraction chamber/planar column (for extraction and preparative work), a holding device for 20×20 cm thin-layer chromatography (TLC) plates, a variable-speed motor, solvent inlet and outlet. The angle of the rotor can be adjusted. The solvent inlet can be attached to an LC pump or solvent flow can be produced by gravitational force and the solvent outlet can be connected to a fraction collector.

Recently, great attention has been devoted to natural compounds capable of scavenging free radicals and preventing oxidative damage of tissues in degenerative diseases [5–8]. Oak bark is recognized as a rich source of such compounds. It contains plant phenolics such as flavonoids and phenolic acids in a wide range of polarity [9–11]. The main aim of this work was to study ExtraChrom as a tool for preparative use (extraction of plant material and fractionation of the obtained extract), and also for analytical separation and detection of some characteristic compounds. The possibility of fractionation of oak bark crude extract towards isolation as well as qualitative analysis of catechins in the oak bark crude extract was studied.

2. Experimental

2.1. Plant material

Milled *Quercus robur* L. bark (oak bark) purchased from University Pharmacy (*Q. cortex* lot No. 65/0-2000; Helsinki, Finland) was sieved to fractions with the following medium particle sizes: 0.35 mm (between 0.45 mm and 0.25 mm), 0.6 mm (between 0.75 mm and 0.45 mm), 0.875 mm (between 1.0 mm and 0.75 mm) and >1.0 mm.

2.2. Chemicals

Methanol for the extraction was of technical grade (Exxon, Finland) and was filtered prior to use. All the other chemicals were of analytical grade. Phosphoric acid and vanillin were purchased from Merck (Darmstadt, Germany); 96% ethanol was obtained from Alko (Helsinki, Finland); (+)-catechin, (−)-epicatechin, (±)-catechin, quercetin, quercitrin, rutin, caffeic acid, *p*-coumaric acid, chlorogenic acid, ellagic acid and free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) were from Sigma (St. Louis, MO, USA). Milli-Q purified water was used.

2.3. Preparation of standard solutions

Separate stock solutions (1 mg/ml in methanol) of (+)-catechin and (−)-epicatechin, quercetin, quercitrin, rutin, caffeic acid, *p*-coumaric acid, chlorogenic acid, ellagic acid were prepared. Application solutions (0.1 mg/ml) were prepared by diluting the stock solutions with methanol. Additionally, a standard solution containing (+)-catechin and (−)-epicatechin (0.1 mg/ml of each in methanol) was prepared. All the solutions were kept at +4 °C.

2.4. Medium-pressure solid–liquid extraction of plant material

MPSLE was applied to the extraction of oak bark using 80% aqueous methanol as an extractant. The experiments were performed on a Büchi column (230×36 mm I.D., volume 240 ml) filled with 40.0 g of oak bark for each experiment. An M-6000 HPLC pump (Millipore-Waters, USA) was fitted to the

column. The solvent (90 or 135 ml) was pumped through the column at a flow-rate of 6.0 ml/min. The extracts were collected while additional pumping of the same volume of the solvent after equilibrium time of 60 min, evaporated under reduced pressure and the dry mass of residues were determined.

2.5. Rotation planar extraction of plant material

RPE was carried out with ExtraChrom (Fig. 1). The oak plant material was extracted with 80% (v/v) aqueous methanol. To get comparable conditions for the MPSLE experiments, the same operating variables, medium particle size and volume of solvent, were chosen for the RPE experiments. The planar column was filled with 40.0 g of oak bark using a rotational speed of 1700 rpm. The oak bark plant material was wet with either 90 ml or 135 ml of 80% aqueous methanol at the rotational speed set to 1400 rpm and solvent flow to 6 ml/min. The time needed for the wetting was therefore 15 or 22.5 min. After wetting the planar column was let to equilibrate for 60 min. The extracts were collected after the equilibrium time using the rotational speed of 1700 rpm, evaporated to dryness under reduced pressure at 35 °C on water bath and the dry mass of residues were determined.

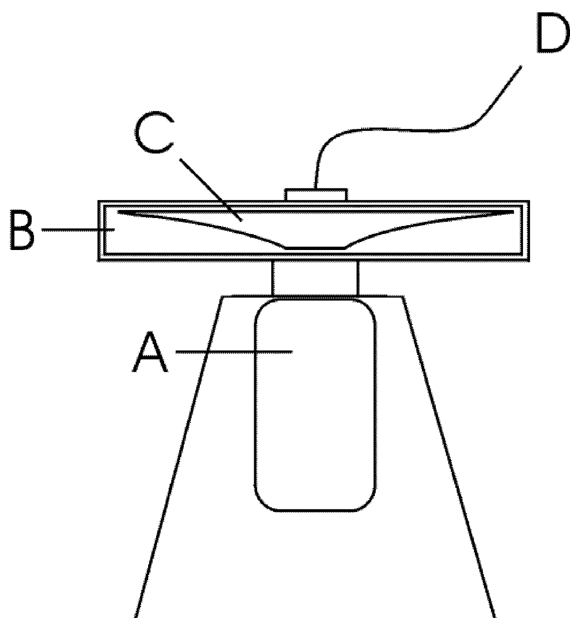

Fig. 1. The schematic figure of the ExtraChrom instrument. A= Motor; B=chamber; C=extraction chamber/rotation planar column; D=tubing from solvent reservoir [3].

2.6. Fractionation by preparative rotation planar chromatography

Fractionation was performed by ExtraChrom, which was dry filled by 56 g of silica gel H for TLC (Merck article 107736), while rotating at 2000 rpm. An 840-mg amount of crude extract was dissolved in 10 ml of ethyl acetate–methanol (1:1, v/v) and applied to the middle of the planar column at a rotation speed of 400 rpm. Thereafter the plate was rotated at 2000 rpm for 10 min and then stopped. After filling the hole in the center of the plate with 10 ml of eluent I (toluene–ethyl acetate–formic acid, 35:15:1, v/v), rotation at 1000 rpm was applied and continued to the end of the fractionation using 400 ml of eluent I. The flow-rate was 6.0 ml/min. The rotating plate was in horizontal position. The eluate was collected manually in tubes, 13 ml in each one. Elution was continued with 100 ml of eluent II (ethyl acetate–methanol, 1:1, v/v), followed with 100 ml of eluent III (methanol). When the eluate excised to flow, the rotation was also stopped.

The content of the tubes was immediately screened by conventional TLC. Solutions with the same composition were combined and concentrated on the rotary evaporator to about 10 ml and again analyzed by TLC. Seven final fractions were evaporated to dryness and weighed.

2.7. Thin-layer chromatography

TLC was performed on precoated silica gel 60, 20×20 cm TLC plates (Merck article 5721), with a 0.25 mm sorbent layer. Original plates were cut into 20×10 cm or 10×10 cm.

2.7.1. Screening of oak bark crude extracts

A 4.0-μl volume of oak bark crude extracts obtained with RPE and MPSLE were applied as 4 mm bands, 15 mm from the left edge of the plates, 10 mm from the bottom of plates, 5 mm apart, with the speed of 10 s/μl by means of Linomat IV

applicator (Camag, Switzerland). Plates (20×10 cm) were developed in normal unsaturated chamber (for 20×20 cm plates) with developing solvent consisting of ethyl acetate–water–formic acid (85:15:10, v/v) [12]. The TLC plates were developed to a distance of 6 cm.

2.7.2. Screening of the collected eluates in tubes and fractions

A 10-μl volume from each tube and up to 25 μl of each of the seven final fractions was applied as 5 mm bands, 10 mm from the left edge of the plates, 10 mm from the bottom of the plates, 3 mm apart, with the speed of 8 s/μl. TLC plates with samples from the tubes were developed as described above. TLC plates for screening of fractions were developed in twin trough unsaturated chamber for plates 10×10 cm, 10 ml of developing solvent (*n*-hexane–ethyl acetate–formic acid, 20:19:1, v/v) in one trough. These TLC plates were developed to a distance of 8 cm.

2.7.3. Separation of epimeric flavan-3-ols (+)-catechin and (−)-epicatechin

Separation was performed on glass-backed 20×20 cm cellulose TLC plates with 0.1 mm layer thickness (Merck article 5716). The plates were cut into 10×10 cm. A 2-μl volume of solutions of standards and oak bark crude extract was applied onto the TLC plates as 10 mm bands, 10 mm from the bottom of the plates and 15 mm from the left edge of the plates, 5 mm apart with the application speed of 12 s/μl. The plates were developed to a distance of 5 cm in normal unsaturated chamber for 10×10 cm plates (Camag) with water as developing solvent.

2.8. Analytical rotation planar chromatography

RPC was performed using ExtraChrom on 20×20 cm TLC cellulose plates (Merck article 5716), with a 0.1 mm sorbent layer. Standard solutions of (+)-catechin, (−)-epicatechin, a mixture of them, a sample of oak bark crude extract, and an oak bark crude extract with the addition of (−)-epicatechin (0.1 mg/ml of each in 80% aqueous methanol) were applied at the speed of 12 s/μl. The application volume was 2 μl.

The separation was performed in the ultra-micro rotation planar chromatographic (U-RPC) chamber, which means that the TLC plate to be analyzed was covered by a glass plate of the same size. The developing solvent, water, was pumped into the center of the TLC plate (via a small hole in the covering glass plate) and the rotation speed of the plate was kept at 1300 rpm. The solvent flow was 0.06 ml/min, developing time 12 min and developing distance 4 cm.

2.9. Detection and documentation

2.9.1. Detection

(1) Natural fluorescence at 366 nm (for flavonoids and phenolic acids).

(2) Dipping reagent: modified vanillin–H_3PO_4 reagent [12–14] (for catechins, proantocyanidins): 10 ml of orthophosphoric acid was added to a solution of 1 g of vanillin in 70 ml of ethanol. The developed plates were dried in a stream of warm air for 2 min and then immersed for 1 s into vanillin–H_3PO_4 reagent by means of Camag immersion device II. Drying in a stream of warm air for 2 min furnished colored bands for separated compounds.

(3) Spraying reagent for antioxidants DPPH [15,16]: 40 mg of DPPH was dissolved in 100 ml of methanol.

2.9.2. Documentation

Documentation of all TLC plates was performed by a Camag Video Documentation System, coupled to a Reprostar 3 transilluminator and a frame grabber system equipped with a 3×1/2 in. charge-coupled device (CCD) camera (Model HV-C20, Hitachi Denshi, Japan) (1in.=2.54 cm). The Video Documentation System was operated with VideoStore 2 V2.30 software.

3. Results and discussion

3.1. Comparison of RPE and MPSLE

The effect of two operating variables, medium particle size of plant material and volume of extractant, on the efficiency of RPE and MPSLE of flavonoids from oak bark was investigated by determining the extraction yield of each individual

Table 1
The effect of extraction variables on the efficiency of the extraction of 40.0 g of oak bark with RPE and MPSLE

Medium particle size (mm)	RPE		MPSLE	
	$m_{extract}$ (g) (V_{MeOH}=90 ml)	$m_{extract}$ (g) (V_{MeOH}=135 ml)	$m_{extract}$ (g) (V_{MeOH}=90 ml)	$m_{extract}$ (g) (V_{MeOH}=135 ml)
0.35	4.3	4.7	4.8	6.0
0.6	3.3	4.4	3.8	4.2
0.875	3.2	3.8	3.6	3.8
>1.0	3.0	–	3.5	–

experiment and by inspection of TLC plates at 366 nm. The operating variables used for the extraction experiments with 40.0 g of oak bark are summarized in Table 1. As is evident from Table 1, oak bark extraction with both extraction techniques proved to be influenced by the medium particle size of plant material. In all cases experiments with the smallest medium particle size of plant material gave the highest extraction yield. Additionally, for both extraction techniques an increase of the solvent volume (from 90 to 135 ml) increased the extraction yield. Qualitative TLC analysis of extracts proved that there is no difference between extracts. The most efficient conditions for the extraction of oak bark were a medium particle size of 0.35 mm and solvent volume of 135 ml and crude extract obtained at these conditions was further fractionated by preparative rotation planar chromatography.

3.2. Fractionation by preparative rotation planar chromatography

TLC experiments with different developing solvents and standards showed that compounds present in the obtained fractions of the oak bark (simple phenolic acids, catechins, quercetin) could migrate and be separated by means of rather unpolar developing solvents such as the combination of *n*-hexane–ethyl acetate–formic acid. A compound can be eluted from the stationary phase on ExtraChrom if it has an R_F value between 0.2 and 0.5 in conventional TLC when developed with the same developing solvent [17]. Because of its lower volatility, toluene was chosen for the eluting solvent mixture instead of *n*-hexane used in TLC experiments and a final eluting solvent with less solvent strength was elaborated to start the preparative separation on silica gel (see Experimental). Mass distribution in seven final fractions is presented in Table 2. The total yield of the whole fractionation relative to the applied mass of the crude oak bark extract was 62% (519.6 mg/840 mg), and as expected, most of the eluted material appeared in the last fraction obtained with eluents II and III. It could be seen in TLC analysis that in fraction 1 at least eight compounds with natural fluorescence of different colors were present. They did not exhibit radical scavenging activity (DPPH reagent). Fraction 2 contained at least nine compounds, mostly different than fraction 1. Some of them showed some radical scavenging activity.

Table 2
Results of fractionation of oak bark extract by preparative rotation planar chromatography

Fraction	Combined tubes	Mass (mg)	%[a]	Remarks
1	1–3	10.5	2.0	
2	4–7	15.0	2.9	
3	8–12	8.2	1.6	
4	13–25	16.2	3.1	
5	26–29	6.7	1.3	(+)-Catechin
6	30	10.8	2.1	(+)-Catechin, leucocyanidin?
7	31–35	452.2	87.0	Mixture of polar compounds

[a] Percentages in the table are calculated relative to the total mass of fractions and not to the mass of the dry extract applied.

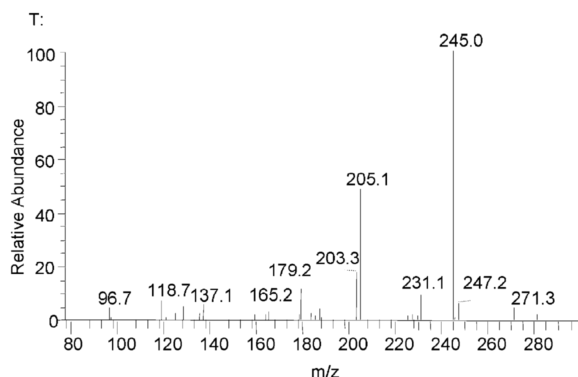

Fig. 2. MS–MS spectra obtained by direct injection of solution of the isolated compound into the LCQ Finnigan MAT mass spectrometer; atmospheric pressure chemical ionization source (negative ionization).

TLC separation of fraction 4 on silica gel using *n*-hexane–ethyl acetate–formic acid (20:19:1, v/v) as developing solvent gave a band of the main compound with a strong natural bright blue fluorescence at 366 nm and a very strong radical scavenging activity, which is characteristic for phenolic acids. The compound lying under (+)-catechin on the TLC plate developed in ethyl acetate–water–formic acid (85:15:10, v/v) could be flavan-3,4-diol leucocyanidin because it gave a red color with the anisaldehyde reagent [18].

After combination of fractions according to TLC analysis, evaporation to dryness gave 6.7 mg of white powder for fraction 5 (Table 2). On silica gel TLC plates with ethyl acetate–water–formic acid (85:15:10, v/v) as developing solvent, the compound from the isolated white powder migrated as (+)-catechin, (−)-epicatechin and some simple phenolic acids. It did not fluoresce at 366 nm on the TLC plate, but gave red coloration with vanillin detection reagent. The isolated compound scanned by MS had molecular mass peak at m/z 290 and tandem mass spectrometry (MS–MS) (Fig. 2) gave the same fragments as standards of (+)-catechin or (−)-epicatechin. Surprisingly, a TLC separation on the cellulose plate using only pure water as developing solvent served for distinguishing between both possible epimers and final identification of the isolated compound as (+)-catechin (Fig. 3a).

Catechin was also present in fractions 4 and 6, but in the mixture with the other unknown compounds. Only in fraction 5 no additional compound could be detected at TLC conditions described under ''screening of the collected eluates'', applying 10 μg of the

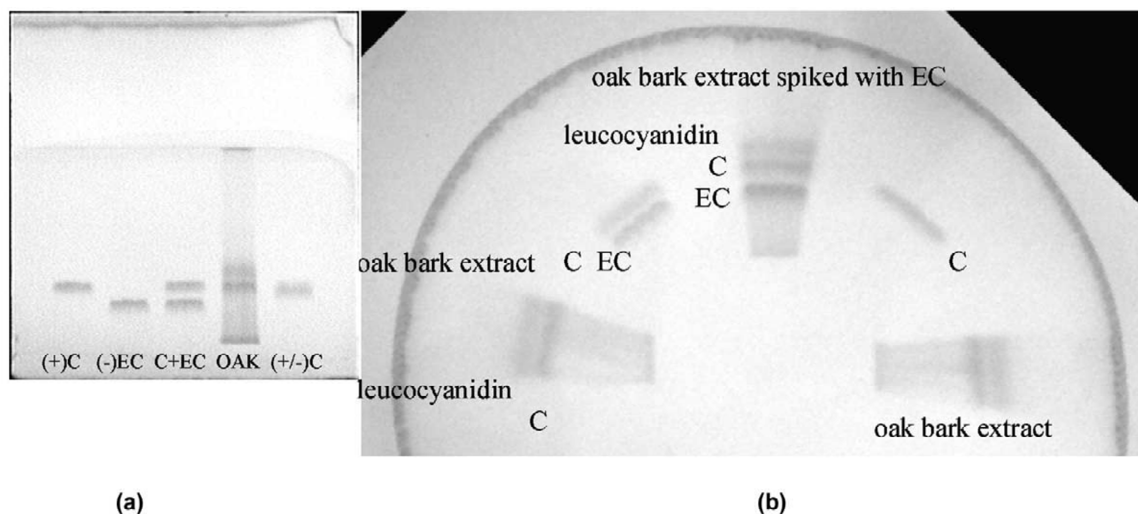

Fig. 3. (a) Separation of (+)-catechin (C) and (−)-epicatechin (EC), on cellulose TLC plates developed in normal developing chamber, using water as developing solvent. (b) Separation of C and EC on cellulose TLC plates developed in U-RPC chamber using water as developing solvent. Oak bark extract was spiked with EC.

fraction. Isolated amount of catechin in fraction 5 was estimated to be 0.8 mg/g of oak bark plant material.

3.3. Separation by analytical rotation planar chromatography

Furthermore, attempts to evaluate the use of the same instrument for analytical RPC separations, qualitative determination of catechins in the oak bark crude extract was performed. For the analytical separations commercially available 20×20 cm TLC cellulose plates were used. The plate was placed as such on the holding device, without any pre-treatment or special procedure before the separation. The samples could be applied as spots by capillaries or bands by an automatic application device (as in this study) or in the form of a circle by hand holding syringe, while the plate is rotating. Also the vapor phase could be adjusted. RPC separation on cellulose plates for the separation of (+)-catechin and (−)-epicatechin using water as the development solvent was carried out in accordance with the achieved linear TLC separation. Several conditions, e.g., suitable solvent flow-rate that depends on the rotational speed used, stationary phase, layer thickness (0.1 mm of cellulose), the viscosity of the mobile phase and the application position (distance from the center of the plate) were adjusted to find the best separation. It was found that pre-washing of the TLC plate, that was necessary for separations of the same compounds in conventional TLC [19], was not needed for the ultra-micro chamber RPC. The impurities or more probably substances from the process of production of plates were equally distributed in the cellulose layer and migrated with the solvent front. Different combinations of rotation speed (from 500 to 1800 rpm) and solvent flow (1 to 0.06 ml/min) were tested as well as the period of time when the solvent was pumped into the center of the TLC plate. When the solvent flow-rate or the rotation speed was too high the solvent did not penetrate into the layer but migrated on the surface of the layer. The successful separation (Fig. 3b) was achieved at conditions stated in the experimental part. (−)-Epicatechin was added to the oak bark extract, since comparison of the circular separation was more difficult than that obtained on conventionally developed plates.

4. Conclusions

The ExtraChrom instrument is the only multifunctional instrument, where solid–liquid extraction and off-line analytical and micropreparative, as well as on-line preparative solid–liquid chromatographic methods can be carried out. Sample-preparation methods used for solid samples have recently been reviewed containing details of traditional methods such as sonication, homogenization, vortex-mixing, percolation and Soxhlet extraction, and some more modern methods, e.g., supercritical fluid extraction, microwave-assisted extraction, pressurized-fluid extraction, forced-flow solid–liquid extraction and automated extraction methods [4]. Using oak bark and *Allium cepa* L. [20] as examples, it can be concluded that the ExtraChrom instrument offers several advantages in the extraction of complex matrices like rapid filling of the extraction chamber, extraction of materials of small particle size, the obtained extract is particle-free, extraction takes place in a closed chamber, and possibility to extract the material successively with solvents of different polarity. In the implementation of solid–liquid extraction and extraction strategy as delineated by Nyiredy [21–23] the RPE method with the ExtraChrom separation instrument seemed to be well suited for screening purposes: 20–50 g of plant material at a time are to be extracted and the number of these samples is fairly large MPSLE proved to be an exhaustive extraction method and the possibility of scaling up the extraction process makes it a suitable method for larger scale preparative extractions [24].

The advantages of this instrument in the preparative separations of complex mixtures were easy and rapid filling of the planar column and possibility to use adsorbent material of small particle size. In analytical separations the possibility to use normal commercially available TLC plates (free choice of stationary phase), the adjustable volume of vapor phase and the optimization of developing solvent to be performed by conventional TLC are beneficial. Also the analytical RPC separations can be used to

scale-up the on-line preparative RPC to be performed on the same instrument.

This paper demonstrates the versatility of various planar separation processes using forced-flow techniques in the extraction, purification and isolation of natural products carried out with a single instrument.

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