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Short communication

Preparative separation of phenolic compounds from *Picea abies* by high-speed counter-current chromatography

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Abstract

An improved method is presented for the isolation of phenolic compounds from needles of Norway spruce (*Picea abies*, Pinaceae). High-speed counter-current chromatography (HSCCC) has been employed as a key step in the semipreparative-scale separation and purification of 26 phenolics from juvenile and mature needles. The isolation of the three 3-*O*-(6″Ac)glucosides syringetin, isorhamnetin and kaempferol is reported together with other flavonols, dihydroflavonols, catechins, stilbenes, and other phenolics.

1. Introduction

Counter-current chromatography (CCC) is a term describing modern liquid–liquid chromatography, without solid support, requiring two immiscible solvent phases [1]. In most reported variants of CCC, one phase remains stationary while the second phase is passed through the stationary solvent phase. The principle of separation involves the partition of a solute between the two phases [2]. High-speed counter-current chromatography, HSCCC, relies on centrifugal force for the retention of the stationary phase [3]. Among the advantages of HSCCC compared to other liquid–liquid techniques are the availability of shorter separation times and wider range of suitable solvent systems [2]. Quantitative recovery of samples is possible [2]. In this work, we used a 6-step process (extraction, liquid–liquid partitioning, adsorbent column chromatography, HSCCC, gel chromatography and preparative HPLC) for the isolation of phenolic compounds from needles of Norway spruce (*Picea abies*). The structure elucidation of 15 flavonols isolated from juvenile needles of Norway spruce by the use of this method, has been reported previously [4]. In addition, we report on the isolation of phenolics previously detected by on-line liquid chromatography–mass spectrometry [5].

2. Experimental

2.1. Crude extracts of needles of Picea abies

Needles were harvested at different sites on the same tree of *Picea abies* (L.) Karst. (Provenance Ukraine) at NISK, Fana, Norway, twice during the growth season 1994. Newly open buds

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(744 g fresh weight) were collected on 16 June, and mature needles (676 g fresh weight) on 20 September. The material was immediately frozen to −20°C and stored at this condition until extraction.

The juvenile and the mature plant material were extracted separately with methanol–water (80:20) three times (3 × 2 h) under constant stirring at room temperature using a total of 10 l of solvent. After concentration at reduced pressure, the water solutions were partitioned against equal amounts of *n*-hexane until the colour of this phase remained unchanged. The crude extracts were applied to a 50 × 4.5 cm glass column (Rotaflo, UK) slurry packed with Amberlite XAD-7 resin (Sigma Chemical Co.). After application of ca. 5 ml concentrated sample, the column was washed with ca. 2 l of distilled water prior to elution of the sample using ca. 600 ml methanol per aliquot.

2.2. High-speed counter-current chromatography (HSCCC)

Preparative-scale separations were performed at room temperature with a Coil Planet Centrifuge 1000, PTR (Pharma-Tech Research Corp., Baltimore, MA, USA) equipped with a 660-ml coil system, an SSI 300 LC pump and an injector. A UV detector (Knauer, 100 mV) measuring at 254 nm was connected to the system together with a Model 600 chart recorder (W&W Scientific, Basle, Switzerland). The coil was filled with a 1:1 equilibrated combination of the upper and the lower phase of the system chloroform–methanol–isopropanol–water (5:6:-1:4), whereas the lower phase was used as the starting mobile phase [2]. The experimental data for the HSCCC given in Table 1 were obtained at room temperature. The juvenile needle extract was eluted in two portions: firstly 2.35 g dry extract was dissolved in 20 ml upper- and lower phase, respectively, and secondly 1.25 g dry extract was dissolved in 10 ml of each of the two phases. Fractions were collected by an Ultrorac 7000 automated fraction collector (Pharmacia, Bromma, Sweden). After 6.8 h and 8.8 h for the two elutions, respectively, the mobile phase was changed from the lower phase to the upper phase of the solvent system. The mature needle extract, 5.45 g dry weight, was dissolved in 30 ml of each of the two liquid phases. Fractions were combined according to similar retention factors (R_F) and visible and fluorescent colours under

Table 1
Experimental data for high-speed counter-current chromatography of extracts of juvenile and mature needles of Picea abies

5Juvenile	Mature		
	Sample 1	Sample 2	
Amount of extract (g)	2.35	1.25	5.45
Amount of solvent used to apply on the column (ml)	40	20	60
Size of injection loop (ml)	60	60	100
Fraction size (ml)	16	16	15
Flow-rate (ml/min)	4	4	3
Spin-rate (rpm)	1000	1000	1000
Total time for each run:			
· Normal phase (h)	6.8	8.8	10.8
· Reversed phase (h)	2.0	2.1	5.0
· Total both phases (h)	8.8	10.9	15.8
Total number of fractions	20 (13 + 7)	20 (13 + 7)	17 (14 + 3)
Sample recovery[a] (%)	94.9		–

[a] The fractions from the two applications of the juvenile needle extracts were combined before evaporation giving the combined tabulated sample recovery. The sample recovery of the mature needle extract is unknown.

UV lamps (254 and 360 nm) after TLC elution (Silica plates, 60 F_{254}, 0.2 mm, Merck, Darmstadt, Germany) using the lower phase of the solvent system as developing solvent.

2.3. Gel filtration

A glass column (52 × 2.6 cm I.D.) was slurry packed with Sephadex LH-20 (Pharmacia, Sweden) to give a column height of 42.5 cm. Fractions from HSCCC, mainly in the range 50–500 mg, were eluted using methanol (HPLC grade) at a flow-rate of 1.67 ml/min. Fractions were collected every 6 min using an automated fraction collector (ISCO Retriever II).

2.4. Preparative HPLC

As a final clean-up, samples from gel filtration were eluted using preparative reversed-phase HPLC (250 × 20 mm I.D., 7 μm RP-18, Macherey-Nagel) (Shimadzu LC-8A pump). Isocratic elution was performed using 18–50% methanol or acetonitrile in water. The flow-rate was in the range 5–12 ml/min, and the effluent was monitored on a UV detector at 280 nm (Pharmacia 2151 UV detector, Bromma, Sweden) connected to a recorder (Pharmacia 2210, Bromma, Sweden).

3. Results and discussion

A refined, 6-step process (extraction, liquid–liquid partitioning, adsorbent column chromatography, high-speed CCC, gel filtration and preparative HPLC) has been developed and applied to the isolation of phenolic compounds from needles of Norway spruce. The enriched methanolic extracts were first partitioned against *n*-hexane, which removed both lipids and most of the pigments (chlorophylls, carotenoids). Secondly, the non-ionic polymeric adsorbent chromatography removed sugar residues and other non-phenolic hydrophilic compounds. However, we employed high-speed counter-current chromatography as the key step in the initial semi-preparative-scale separation and purification of the phenol-rich methanolic needle extract. The choice of solvent system for the HSCCC procedure was based on the results of TLC tests according to Ref. [2]. Upon elution of crude extracts on silica TLC plates in the organic part of the two-phase solvent system, the retention factors were mainly below 0.60. In addition suitability of the solvent system was qualitatively established by investigating the distribution of the sample between a small amount of the two phases.

A group of rather rare acetylated flavonol glucosides were eluted after ca. 3 h during the normal-phase HSCCC elution of the juvenile needle extract. The chromatographic peaks 1 [isorhamnetin 3-*O*-(6″-Ac)glucoside and syringetin 3-*O*-(6″-Ac)glucoside] and 2 [kaempferol 3-*O*-(6″-Ac)glucoside], were clearly separated from the following bands, and the use of HSCCC hence proved to be an appropriate step in the isolation of these flavonols from other non-acetylated flavonols (Fig. 1, upper left). A similar separation, although in reversed order of elution, was observed for these three compounds when eluted on a C_{18} HPLC column [5]. Due to the fact that they differ by only one methoxyl group, the compounds in HSCCC peak 1 are eluted simultaneously on RP-HPLC, and are denoted as a "critical pair" of flavonoids [6]. The retention mechanism on C_{18}-column material is thought to be partition rather than adsorption [7], and a comparison of the retention order in HSCCC and RP-HPLC of the flavonols eluted in the normal-phase mode (HSCCC) of the juvenile needle extract seems to support the theory that both methods rely on the same basic retention mechanism. However, deviations are observed when turning from normal- to reversed-phase elution.

The use of HSCCC alone did not prove to be sufficient as a total isolation and clean-up procedure for any of the present compounds. However, several of the larger chromatographic bands appeared to contain one major component, e.g. band 4 eluted in the normal-phase mode for the juvenile needle extract (Fig. 1, upper left). This band contained kaempferol 3-*O*-glucoside, which is reported to be the main

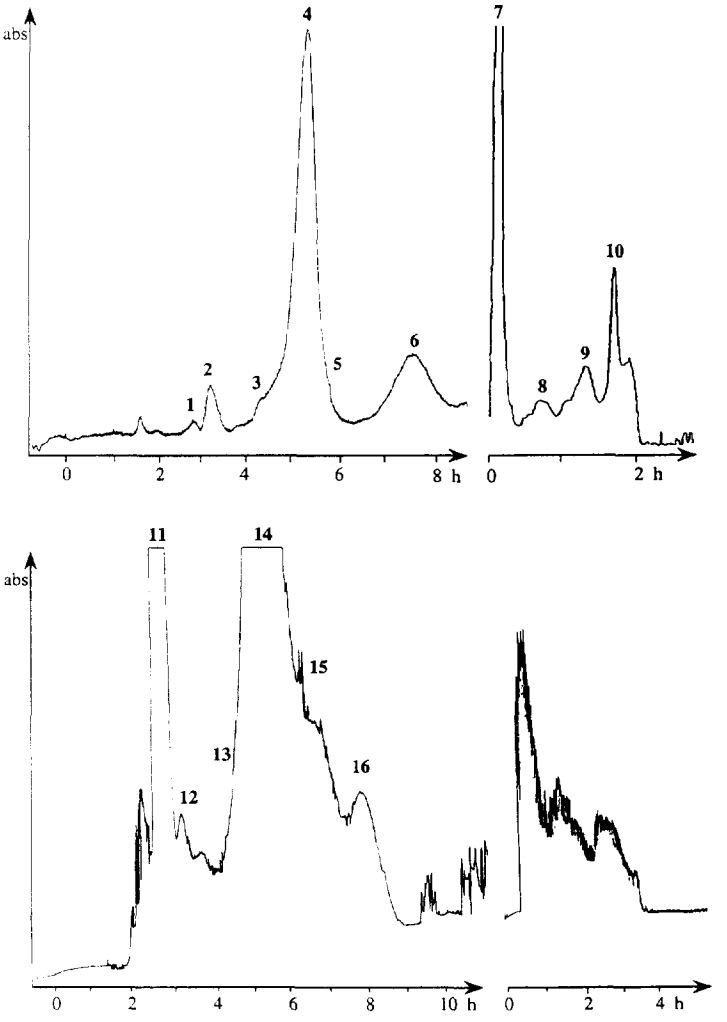


Fig. 1. High-speed counter-current chromatography (HSCCC) of crude extract of needles of Norway spruce (*Picea abies*). Upper chromatograms show the elution of the juvenile needle extract in normal-phase mode (left) and reversed-phase mode (right). Lower chromatograms show similar elutions of a mature needle extract. The numbered regions (bands) correspond to the elution of the compounds in Table 2. The reversed-phase elution of the mature needle extract contained mainly tannins, which were neither purified nor their structures elucidated.

flavonol in Norway spruce needles [8]. In the reversed-phase part of this chromatogram (Fig. 1, upper right), myricetin 3-*O*-glucoside was eluted as the main constituent of band 9 whereas taxifolin 7-*O*-glucoside appeared in band 10. The chromatogram obtained for the mature needles in the normal-phase mode gave *p*-hydroxyacetophenone in band 11 and its glucosidic analogue, picein, in band 14, these compounds appearing as two of the major phenolic constituents in this extract (Table 2). *E*-Isorhapontin and coniferin, two other compounds found in relatively large amounts, were eluted prior to picein giving peak overlapping in the chromatogram (band 13; Fig. 1, lower left). The large amounts of compounds eluted in the reversed-phase elution of the mature needle extract (Fig. 1, lower right), gave only low R_F values when applied to silica TLC in the lower phase of the solvent system chloroform–methanol–isopropan-

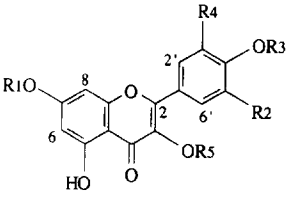
Table 2
Flavonoids and phenolics isolated from needles of Norway spruce by means of high-speed counter-current chromatography and other chromatographic techniques

Band	Compound	Mass
Juvenile needles		
1	Syringetin 3-(Ac)-glucoside	0.3
1	Isorhamnetin 3-(Ac)-glucoside	1.0
2	Kaempferol 3-(Ac)-glucoside	7.8
3	Isorhamnetin 3-glucoside	5.5
4	Kaempferol 3-rutinoside	8.3
4	Isorhamnetin 3-rutinoside	2.1
4	Kaempferol 3-glucoside	125.5
4	Kaempferol 7-glucoside	27.2
5	Laricitrin 3-glucoside	1.6
6	Quercetin 3-glucoside	23.3
7	Catechin	60.1
7	Epicatechin	1.2
8	Myricetin 3-rutinoside	3.5
9	3'-O-Caffeoyl-quinic acid	3.0
9	Kaempferol 3,4'-diglucoside	6.3
9	Quercetin 3-rutinoside	1.7
9	Myricetin 3-glucoside	67.5
10	Laricitrin 3-rutinoside	4.1
10	Taxifolin 7-glucoside	8.7
Mature needles		
11	*p*-Hydroxyacetophenone	275.0
11	Catechin	60.9
12	Taxifolin	2.9
13	E-Isorhapontin	94.1
13	Coniferin	107.4
14	Picein	113.1
14	E-Piceid	5.4
14	Isorhamnetin 3-glucoside	4.5
15	E-Astringin	–
16	Kaempferol 3,4'-diglucoside	–

The numbering corresponds to the assigned chromatographic bands in Fig. 1. Masses of totally purified compounds are given in milligram.

ol–water (5:6:1:4), and the spots gave a red colour reaction when a solution of vanillic acid was applied to the TLC plate. This is in accordance with the chromatographic behaviour and colour reaction of tannins [9].

The Sephadex LH-20 gel material is designed to separate mixtures predominantly on the basis of their molecular size, the larger molecules being eluted first [9]. However, since the LH-20 material also separates by adsorption and normal-phase partitioning, it allows better separation in the isolation of flavonoids when a step gradient of methanol and water is used as solvent [10]. In order to avoid time-consuming evaporation processes for fractions rich in water, we used pure methanol as eluent for the LH-20 column. Catechin and myricetin 3-*O*-glucoside were completely separated and purified from the juvenile needle extract by a combination of HSCCC and gel filtration. However, due to the very diverse nature of the phenolic compounds in the extracts, we experienced considerable chromatographic band overlapping in the HSCCC and gel filtration part of the isolation procedure. Thus, the majority of the compounds from HSCCC were submitted to both gel filtration and preparative RP-HPLC as a final clean-up procedure. In this way, 15 flavonols, 2 dihydroflavonols, 2 catechins, 3 stilbenes, and 4 other phenolic compounds were isolated from juvenile and mature needles of Norway spruce (Table 2, Figs. 2 and 3).



Compounds	R1	R2	R3	R4	R5
Kaempferol 3,4'-diglc	H	H	Glc	H	Glc
Myricetin 3-rut	H	OH	H	OH	Rut
Myricetin 3-glc	H	OH	H	OH	Glc
Quercetin 3-rut	H	H	H	OH	Rut
Quercetin 3-glc	H	H	H	OH	Glc
Laricitrin 3-rut	H	OH	H	OMe	Rut
Laricitrin 3-glc	H	OH	H	OMe	Glc
Kaempferol 7-glc	Glc	H	H	H	H
Kaempferol 3-rut	H	H	H	H	Rut
Isorhamnetin 3-rut	H	H	H	OMe	Rut
Kaempferol 3-glc	H	H	H	H	Glc
Isorhamnetin 3-glc	H	H	H	OMe	Glc
Kaempferol 3-(6"Ac)glc	H	H	H	H	(Ac)Glc
Syringetin 3-(6"Ac)glc	H	OMe	H	OMe	(Ac)Glc
Isorhamnetin 3-(6"Ac)glc	H	H	H	OMe	(Ac)Glc

Fig. 2. Structures of flavonols isolated mainly from juvenile needles.

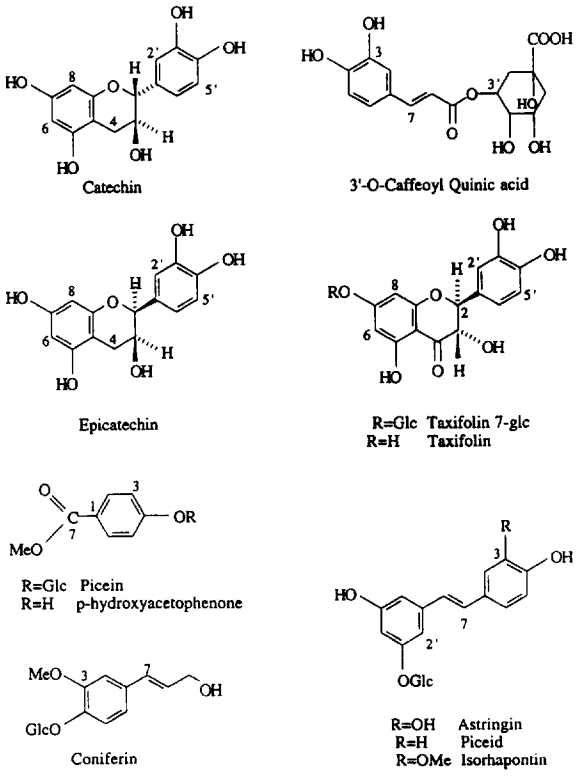


Fig. 3. Structures of phenolics isolated mainly from mature needles.

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