Journal of Chromatography, 538 (1991) 193–202
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 685

Applications of high-speed counter-current chromatography for the separation and isolation of natural products

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ABSTRACT

High-speed counter-current chromatography (HSCCC) with a Ito multi-layer coil separator–extractor was applied to perform efficient separations of natural products and to isolate pure compounds from crude plant extracts. The high performance of the system was demonstrated in the separation and purification of natural antioxidants from rosemary and sage extracts, bitter principles from hops and aroma-relevant constituents, such as phthalides, from celery and parsley roots. In addition, the evaluation of a gas chromatographic-compatible non-aqueous solvent system, suitable for the separation of flavour extracts, is described.

INTRODUCTION

In recent years there has been a renaissance of liquid–liquid chromatographic (LLC) methods, especially in the separation of natural products. These developments mainly involved the emergence of different counter-current chromatographic (CCC) techniques [1]. Further, the advent of high-speed CCC (HSCCC) led to considerable improvements in terms of separation efficiency, analysis time and handling of the apparatus compared with, *e.g.*, droplet counter-current chromatography (DCCC) [2–5].

In this paper we report investigations with an Ito multi-layer coil separator–extractor (a centrifugal CCC system) and several successful applications concerning the preparative isolation of antioxidant principles [6,7] as pure compounds from crude rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) solvent extracts and describe the evaluation of a novel non-aqueous biphasic system with good hydrodynamic properties which permits the preparative isolation of bitter principles from hops and aroma-relevant constituents from celery (*Apium graveolens* L.) and parsley [*Petroselinum crispum* (Mill.) Nyman syn. *P. sativum* Hoffm.] roots.

Additionally, it is shown that the present method has the advantage over, for example, adsorption chromatographic methods that the artefact formation of

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0021-9673/91/$03.50

sensitive compounds is prevented and therefore compounds could be isolated that were not detectable by adsorption chromatography [8].

EXPERIMENTAL

All separations were carried out with a multi-layer coil planet centrifuge called the Ito multi-layer coil separator–extractor (obtained from (P.C. Inc., Potomac, MD, U.S.A.), equipped with a 160 m × 1.6 mm I.D. coil and with a total column capacity of *ca.* 325 ml. For solvent delivery the chromatograph was connected to a Gilson 303 single-piston pump with pressure module. The effluent from the outlet of the column was continuously monitored at 254 and 280 nm using a Uvicord III 2089 UV detector (LKB, Bromma, Sweden) connected to an LKB 2210 two-channel recorder, and fractionated into test-tubes with an LKB Ultrorac 7000 fraction collector. Samples were dissolved in the respective mobile phase and injected by means of a six-way valve and a 4.5-ml sample loop.

In each experiment the non-rotating multi-layer coiled column was first entirely filled with stationary phase, then the mobile phase was pumped into the column while the apparatus was run at the optimum speed of 800 rpm. After equilibrium had been reached, as indicated by a clear mobile phase eluting at the column outlet (effluent was collected in a graduated cylinder), the sample solution was injected. The percentage of the stationary phase retained in the column could easily be determined from the volume collected in the cylinder having been displaced by the mobile phase.

The appropriate elution mode used depended on the whether the upper or the lower phase of the biphasic solvent system applied was chosen as the stationary phase. When the stationary phase was the lower phase, mobile phase (upper phase) was pumped into the "tail" end of the coil (tail-to-head elution mode), and when the mobile phase was the lower phase, it was pumped into the "head" end of the coil (head-to-tail elution mode). The two-phase solvent mixtures applied for the different separations were thoroughly equilibrated in a separating funnel at room temperature and separated before use.

Monitoring of the fractions of interest was carried out by high-performance liquid chromatography (HPLC). The HPLC system consisted of a Gynkotek 300B double-piston pump, a Gynkotek 250B low-pressure gradient former and a Philips PU 4021 diode-array detector, connected to a Shimadzu CR-3A integrator. HPLC separations were performed on a 250 mm × 4 mm I.D. LiChrospher 100 RP-18 (5-μm) column (Merck, Darmstadt, F.R.G.).

RESULTS AND DISCUSSION

Separation of antioxidants from rosemary and sage

Various crude solvent extracts of rosemary and sage were resolved by HSCCC with hexane–ethyl acetate–methanol–water (70:30:14:8) as solvent system (Fig. 1). The selection of the three different extracts mentioned in Fig. 1 was based on how far the solvent polarity influences the extraction of the antioxidants. All three extracts showed a strong antioxidative activity [8].

According to a rapid thin-layer chromatographic method for the choice of a two-phase aqueous solvent system that is applicable to the separation of a given

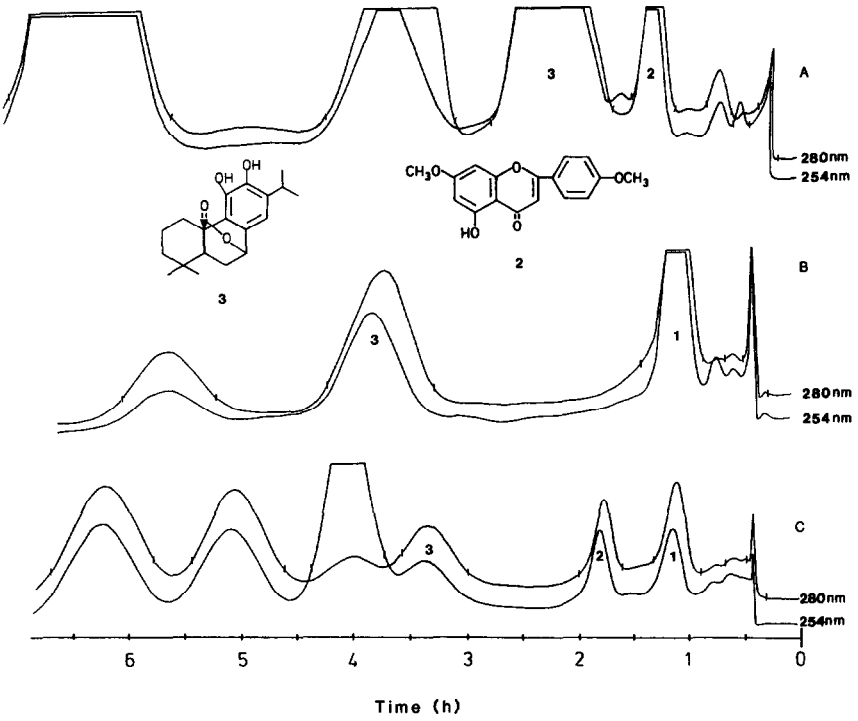

Fig. 1. HSCCC of (A) rosemary methanol, (B) sage hexane and (C) rosemary pentane extracts. Solvent system, hexane–ethyl acetate–water–methanol (70:30:8:14); flow-rate, (A) 1.6, (B) and (C) 1.5 ml/min; rotational speed, 800 rpm; sample, (A) 715, (B) 750 and (C) 235 mg. Peaks: 1 = unknown; 2 = genkwanin 4′-methyl ether; 3 = carnosol.

mixture or extract, described by Hostettmann *et al.* [9], the chosen solvent mixture appeared to be the most suitable, particularly in terms of separation efficiency. The separations were performed in the tail-to-head elution mode, whereby the lower phase was used as stationary phase and the upper phase was eluted through the column at flow-rates of 1.5 and 1.6 ml/min.

As shown in Fig. 1, the applied chromatographic conditions facilitate the separation of co-extracted spice constituents such as chlorophylls and carotenoids (the first three peaks in all chromatograms) from the relevant antioxidants, which makes it possible to isolate the antioxidant principles, genkwanin 4′-methyl ether (5-hydroxy-7,4′-dimethoxyflavone) (peak 2), carnosol (3) and a still unknown component (1), as pure compounds. Although large amounts of crude extract (see Fig. 1) were charged into the multi-layer coil, the compounds of interest were baseline separated and no peak overlap with other spice constituents occurred. Collecting only the fractions at the peak maximum, for example, the separation of the methanol extract of rosemary in a single run provided carnosol in amounts up to 66 mg and genkwanin 4′-methyl ether up to 6 mg with a purity of *ca.* 95%. The structure of the pure antioxidants was checked by spectroscopic methods (mass, IR, NMR). The spectra obtained were compared

with literature data. The 90% purity of the unknown component (peak 1) enabled us to perform gas chromatographic–mass spectrometric (GC–MS) analyses without further clean-up steps.

The results show clearly that this CCC method facilitates the direct isolation of pure antioxidants from crude spice extracts. Compared with the classical adsorption chromatography it is not necessary to clean up the extract (*e.g.*, by solvent washing, bleaching with active carbon) [10] before isolating the antioxidants. The applied solvent system in combination with a proper elution mode permits the direct efficient separation of undesirable constituents (*e.g.*, chlorophylls and carotenoids) in a single separation step. Apart from this advantage, preparative HSCCC offers the possibility of large sample loads without a decrease in separation efficiency (compare Fig. 1A with Fig. 1C) and provides a sample recovery of up to 99%.

Another aspect concerns the problem of artefact formation of sensitive compounds (*e.g.*, polyphenolic substances like the above-mentioned antioxidants) during work-up procedures. Compared with adsorption chromatographic separations, the lack of a solid support in CCC prevents artefact formation, based on interactions between solute molecules and sorbents. Comparative investigations of the same extract by CCC and adsorption chromatography furnish information about which constituents were originally in the extract and which have to be regarded as work-up artefacts (*e.g.*, produced via adsorption chromatographic separations).

In these studies, the not yet completely identified, highly antioxidative compound that gives peak 1 in Fig. 1 apparently only occurs in non-polar solvent extracts of rosemary and sage [8]. It seems to be a "precursor compound" of the known antioxidants such as carnosol, rosmanol, rosmaridiphenol and carnosic acid [6,10,11]. Different treatments and derivatization reactions of this substance, comparable to the work-up procedures with rosemary extracts described in literature [6,10], revealed the formation of the above-mentioned antioxidants [8]. As this compound could not be isolated or detected in fractions separated by adsorption chromatography on silica gel columns, the applied HSCCC method also appears to be a useful separation technique for investigations concerning artefact formation of sensitive compounds.

Separation of bitter principles from hops

Our interest was to isolate pure humulones from a crude carbon dioxide hop extract for use in metabolic studies. The separation of hop bittering compounds presents a certain analytical problem, as the humulones and lupulones consist of a number of methyl homologues and are closely related in their structures (Fig. 2). In order to achieve a efficient chromatographic separation, we sought an appropriate non-aqueous CCC solvent system, because the solubility of humulone in aqueous solutions is very poor.

Tests on the hydrodynamic properties of different possible two-phase systems according to Ito [4] showed that hexane and acetonitrile, mixed with *tert.*-butyl methyl ether as "modifier", are well suited, and the system hexane–*tert.*-butyl methyl ether–acetonitrile (10:1:10) proved to be promising. The retention of the stationary phase, which mainly determines the quality of a separation [4], was of the order of 80–90% in the reversed elution mode (head-to-tail elution mode).

Fig. 3 shows the chromatogram of a small-scale separation (sample loading *ca.* 24 mg) of bitter principles from the crude carbon dioxide hop extract. The first large

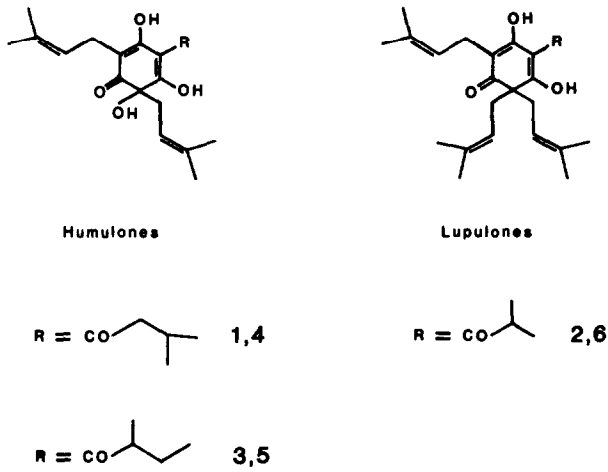

Fig. 2. Structural formulae of important hop bittering compounds. 1 = Humulone; 2 = cohumulone; 3 = adhumulone; 4 = lupulone; 5 = colupulone; 6 = adlupulone.

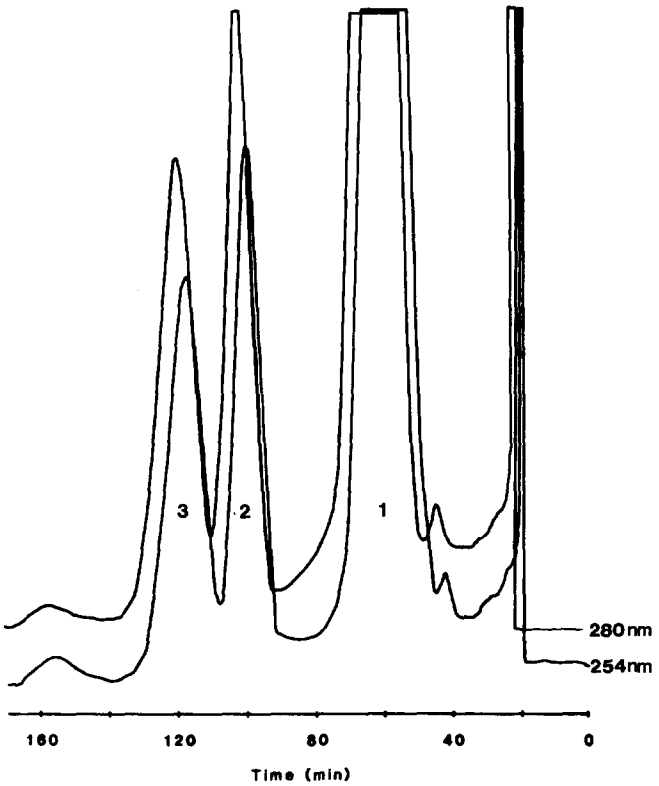

Fig. 3. HSCCC of a hop extract. Solvent system, acetonitrile–*tert.*-butyl methyl ether–hexane (10:1:10); flow-rate, 2.0 ml/min; rotational speed, 800 rpm; sample, 24 mg. Peaks: 1 = cohumulone, humulone, adhumulone; 2 and 3 = lupulones.

peak, marked 1, indicates cohumulone (ascending peak side), adhumulone and humulone, and the following two peaks (2 and 3) show the corresponding lupulones. Although this solvent system only separates groups of constituents (humulones and lupulones), the isolation of pure compounds was achieved. During the first part of a preparative HSCCC run with a sample loading of 500 mg, 1.5-ml fractions were taken, increasing to 5 ml in the last part. The components of these fractions were monitored by reversed-phase high-performance liquid chromatography (RP-HPLC) [12], using pure humulones and lupulones as references. UV detection in the HPLC of the relevant fractions showed only one peak. Although there is only the small difference of a single methyl group, 30 mg of cohumulone of purity >95% and 98 mg of humulone with the same purity could be isolated in a single run. The isolation of pure adhumulone was not tried and seems to be impossible with the configuration used, as it is with virtually any LC system. The separation concerning the lupulones was slightly worse and would require additional optimization of the biphasic system, but this task was beyond the scope of this study.

Compared with HPLC separations of hop extracts, where two RP 18-columns have to be connected in series [13] in order to achieve the separation efficiency required and a solvent is used that contains acid (eluent pH 2.0) to improve the elution profile, which is not desirable for preparative purposes in order to avoid possible acid-catalysed reactions, HSCCC also in this instance provides a mild, time-saving separation technique.

Separation of flavour compounds

A special task in separating flavour substances by LC is to find a system that is compatible with GC, which of course represents the state of the art for the analysis of such components. LC on silica gel, in principle well compatible with GC with respect to the solvents usually applied, is not useful for the separation of more polar compounds, because sample losses can occur owing to irreversible adsorption. Therefore addition of polar modifiers (*e.g.*, methanol) is required at the expense of selectivity and resolution [14]. The widely used reversed-phase chromatography is based, at least for the range of polarities covered by most flavour compounds, on aqueous solvent mixtures, a fact that necessitates an additional extraction step before GC analysis.

Accordingly, the non-aqueous biphasic system hexane–acetonitrile (1:1) was selected with respect to its GC compatibility as a basis for the following optimizations. A set of biphasic systems, obtained by addition of different amounts of the modifiers *tert.*- butyl methyl ether and dichloromethane, was evaluated, using a mixture of test compounds (95% purity) with different functional groups. The components were mixed in equal amounts. For better UV detectability, the aromatic compounds benzyl alcohol, benzyl methyl ketone, benzyl acetate and ethyl benzoate were chosen as test substances. For every phase system the number of theoretical plates N and the resolution R_s were calculated [15]. The results are summarized in Table I.

All separations were carried out in the head-to-tail elution mode with the upper phase as stationary phase. The pure hexane–acetonitrile system shows the maximum difference in polarity between the two phases, resulting in the smallest capacity factors for the test substances used. The system acetonitrile–*tert.* butyl methyl ether–hexane (10:1:10) exhibited good stability (retention of stationary phase) and a good separation power with an average theoretical plate number of 950. Increasing the modifier content

TABLE I

SEPARATION OF A POLAR TEST MIXTURE IN DIFFERENT NON-AQUEOUS BIPHASIC SOLVENT SYSTEMS

Solvent system[a]		Peak No.							
		P-1[b]		P-2		P-3		P-4: N	$\bar{N}$
		N[c]	$R_s(1,2)$[c]	N	$R_s(2,3)$	N	$R_s(3,4)$		
I	A–H (1:1)	1390	2.3	890	2.0	705	4.9	1000	1000
II	A–B–H (10:1:10)	1000	1.6	1025	2.0	865	4.3	900	950
III	A–B–H (10:3:10)	790	0.4	870	1.9	920	3.8	860	860
IV	M–H (1:2)	1085	3.7	1310	2.3	1220	1.7	1210	1205
V	A–D–H (7:3:13)	1180	2.9	1280	1.9	1060	3.9	1380	1225
VI	A–D–H (3:2:7)	Not stable during the run							

[a] A = acetonitrile; B = *tert.*-butyl methyl ether; D = dichloromethane; H = hexane; M = methanol.

[b] For identity of peaks, see Fig. 4.

[c] Theoretical plate number N and resolution between neighbouring peaks $R_s(x,y)$ were calculated according to Conway and Ito [15].

to a ratio of 10:3:10 markedly reduced the hydrodynamic stability and also the separation efficiency. Replacement of the ether modifier with dichloromethane brought a further increase in separation efficiency. The average plate number reached 1225, with clear baseline resolution between the individual peaks (Fig. 4B). The polarities of the two phases were obviously relatively close to the pure hexane–acetonitrile (1:1) system (Fig. 4A), as judged by the rapid elution of the compounds. An only very slight increase in the modifier content, however, led to an enormous deterioration of the stationary phase retention. This effect is even more drastic if the percentages of the solvents in both biphasic systems are compared: system V, 30.5% A, 13.0% D, 56.5% H; system VI, 25.0% A, 16.7% D, 58.3% H.

Solvent system II in Table I was successfully applied for the separation and preparative isolation of phthalides and other sensorially active constituents from celery and parsley roots. The first application was the isolation of phthalides, the main components and responsible for the typical flavour of these roots, in order to obtain pure compounds for reference spectra (NMR, IR). The samples were commercially available celery oils and also extracts that were prepared by cold solvent extraction of plant material which was ground under liquid nitrogen. The above solvent system was used for several preparative HSCCC runs in the reversed elution mode with sample loadings of *ca.* 500–750 mg. In this context it is noteworthy that an increase in the sample loading to 750 mg did not influence the separation of the two groups of phthalides.

Fractions collected at the peak maximum of the relevant peaks (see Fig. 5) provided sedanenolide and sedanolide with a purity of *ca.* 85% in amounts ranging from 1 to 30 mg. 3-*n*-Butyl phthalide and the two butylidene phthalides were isolated with purities of about 60%. With regard to the separation of these constituents the

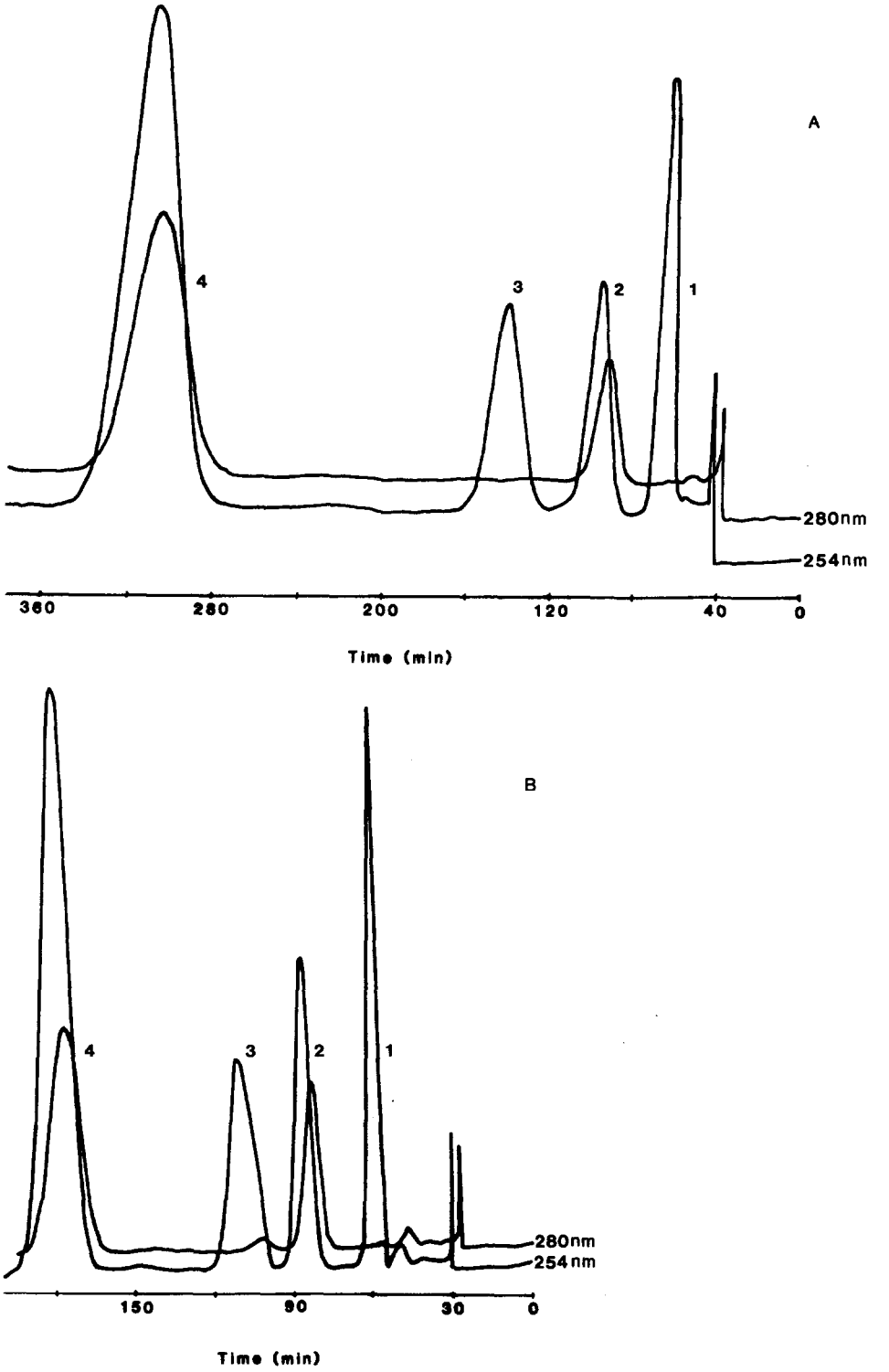

Fig. 4. Separation of (1) benzyl alcohol, (2) benzyl methyl ketone, (3) benzyl acetate and (4) ethyl benzoate by HSCCC. Solvent systems, (A) acetonitrile–hexane (1:1) and (B) acetonitrile–dichloromethane–hexane (7:3:13); flow-rate, 1.5 ml/min; rotational speed, 800 rpm.

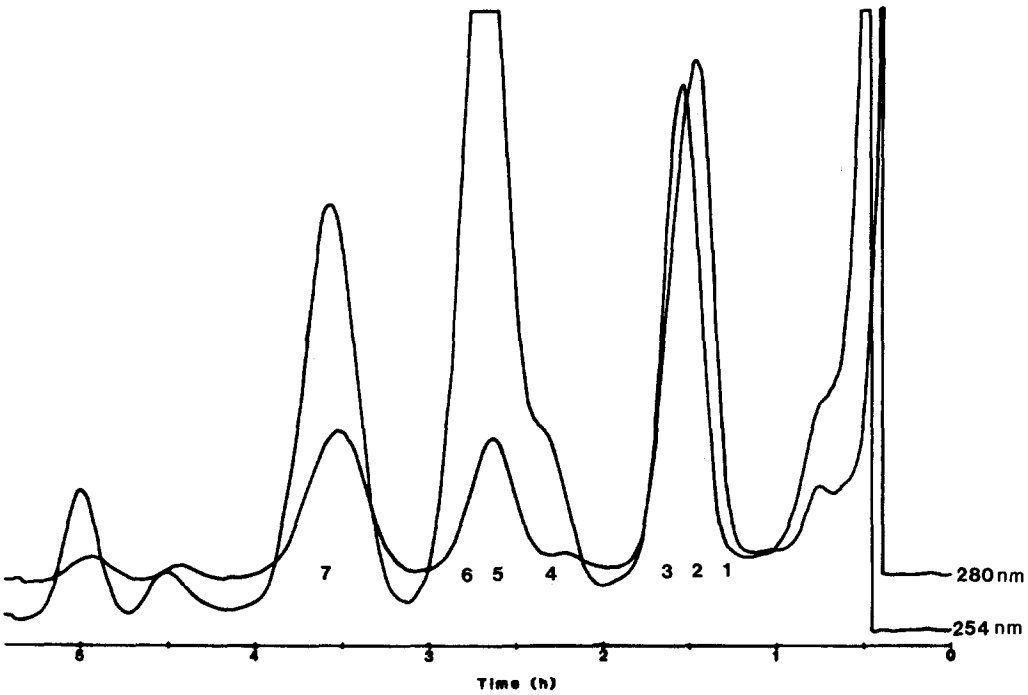

Fig. 5. HSCCC of a commercial celery oil. Solvent system as in Fig. 3; flow-rate, 1.0 ml/min; sample, 200 mg; rotational speed, 800 rpm. Distribution of single components: 1 = celery-like trace component; 2 = sedanenolide; 3 = 3-*n*-butyl phthalide; 4 = (*E*)-butylidene phthalide; 5 = sedanolide; 6 = (*Z*)-butylidene phthalide; 7 = benzyl benzoate.

solvent system has to be optimized. The fractions obtained were simultaneously analysed by GC and GC–MS to identify the components. Structural confirmaton was achieved by comparison with literature mass spectra.

As a consequence of the large amounts of sample being separated in a single run, the localization of sensorially active components in individual fractions was easily performed by checking the smell of the fractions. In this way a trace component (see 1 in Fig. 5) possessing a very strong celery-like odour could be detected. The appropriate fractions were collected and the desired component could be enriched from a barely detectable level in the original oil to a concentration that facilitated subsequent identification and structural elucidation of this substance [16].

In the same way, two unknown C_{17}-polyacetylenic alcohols possessing very similar mass spectra could be isolated from a parsley root solvent extract. The separation of 350 mg of extract was also carried out in the head-to-tail elution mode, using solvent system II in Table I. The first compound, a diol, eluted in fractions 6–9, whereas being relatively polar, the second compound, with only one hydroxyl group, eluted in fractions 50–55 and could be isolated in a yield of 35 mg with a purity of 98%. The structure was elucidated by means of spectroscopic methods such as NMR, FT-IR and mass spectrometry [17].

CONCLUSION

Liquid–liquid chromatographic methods such as DCCC have already demonstrated their potential especially in the field of natural product isolation. The recently developed HSCCC method brought about a great improvement in LLC techniques in terms of separation and partition efficiency and separation time, required for preparative fractionations of complex mixtures. As a consequence of the increased flow and mass transfer rates, however, the appropriate solvent system has to meet the demands of special hydrodynamics involved in the separation process. Therefore, most of the published biphasic systems for LLC have to be re-examined for their ability under HSCCC conditions. A great part of these solvent systems was collected under the special requirements of droplet-formation ability for DCCC, so that many new biphasic systems are conceivable that may be well suited for HSCCC but have never been mentioned in the literature on earlier LLC techniques. On the other hand, great potential for the HSCCC method could lie in the separation of essential oils or flavour extracts, using non-aqueous solvent systems that are compatible with GC such as the described acetonitrile–hexane mixture.

Another important advantage of the CCC techniques is the absence of a solid sorbent. Hence a potential source of complications arising from irreversible sample adsorption and contamination from the sorbent is eliminated. Decompositon or denaturation, or in general artefact formation, is reduced to a minimum, as is well demonstrated by the isolation of the antioxidant principles of rosemary and sage.

In conclusion, HSCCC represents a valuable extension of chromatographic methods, particularly in the separation and purification of natural products.

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