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Combination of supercritical fluid chromatography with thin-layer chromatography on a semi-preparative scale

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ABSTRACT

Semi-preparative supercritical fluid chromatography (SFC) is a very valuable tool for the separation of unknown complex mixtures, as it may allow the isolation of sufficient amounts of individual constituents for ^{1}H and ^{13}C NMR spectroscopic identification. Semi-preparative SFC uses large-diameter packed columns and requires an efficient collection system. The performances of 4.6, 10 and 21 mm I.D. columns were compared and three different collection methods were tested for their efficiency: collection in pressurized stainless-steel tubes or in specially shaped glass vials, and condensation of the effluent at the starting line of a semi-preparative thin-layer chromatographic plate. The good yields that characterize the first two techniques could not be achieved with the third. The plate could, however, be developed, leading to a two-dimensional semi-preparative chromatographic separation. The spots must be localized on the plate in a non-destructive way in order to recover the samples. A general method is proposed, using berberine chloride, and also a more specific method, which allows the selective detection of certain classes of compounds.

INTRODUCTION

During the past 30 years, the development of capillary columns, first for gas chromatography (GC) and subsequently for other chromatographic techniques, has increased the efficiency of separations tremendously. This is also true for newer techniques such as supercritical fluid chromatography (SFC), for which efforts have been mainly directed toward capillary open-tubular and capillary packed columns. Thanks to the low flow-rates of mobile phase that they require, such small-bore columns can easily be coupled to mass or IR spectrometers for compound identification. However, mass or IR spectra do not often permit complete structure elucidations of unknown molecules. On the other hand, ^{1}H and ^{13}C NMR spectroscopy are very valuable identification methods, but they suffer from a lack of sensitivity. The low capacity of capillary columns precludes the use of NMR spectroscopy when encountering unidentified compounds.

There is therefore a real need for powerful semi-preparative separation methods when dealing with complex mixtures of unknown constituents. Large-diameter packed columns possess the high loadability required, but are characterized by higher flow-rates of mobile phase and poorer resolving power than capillary columns. The

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large flow of carrier is particularly troublesome in liquid chromatography, owing to safety considerations, disposal problems and tedious work-up to remove the solvent. In addition, the high flow-rate renders difficult the on-line connection of liquid chromatographs in "hyphenated techniques" [1]. This is very detrimental because combinations of different chromatograhic methods allow the lower efficiency of large-scale separations to be overcome. In this regard, SFC appears very attractive for preparative work as the volatility of CO_2 allows straightforward solvent elimination for either transfer or collection.

In this work, we used a supercritical instrument that can accommodate packed columns of up to 21 mm I.D. Unlike analytical SFC, the semi-preparative mode implies the collection of the compounds after separation. We therefore also compared three different techniques of collection, namely collection under pressure, collection by decompression and deposition on a thin-layer chromatographic (TLC) plate. As a direct extension, a subsequent part is devoted to the coupling of semi-preparative packed-column SFC with semi-preparative TLC. Coupling of SFC with TLC offers the unique advantage of a two-dimensional semi-preparative separation.

The volatility of CO_2 had been extensively exploited for coupling SFC with SFC [2–4], GC [4], high-performance liquid chromatography (HPLC) [5] and TLC [6,7]. However, these coupled techniques remain on an analytical scale at least in one dimension. On the other hand, coupling of HPLC with TLC has been reported several times, but only a low flow of the liquid solvent could be transferred in order to prevent flooding of the plate [8–10]. We have not found any report in which large-diameter packed-column SFC or semi-preparative TLC were coupled with another semi-preparative or preparative technique.

In order to recover the solutes, semi-preparative SFC–TLC separation requires non-destructive methods for the detection of spots. Two methods, general and specific, have been developed and applied to several examples of two-dimensional separations. All analyses led to the isolation of sufficient pure material for NMR analysis.

In the following sections, the term "semi-preparative" refers to separations of amounts of substances ranging from milligrams to tens of milligrams.

EXPERIMENTAL

The samples used were either commercially available chemicals or came from natural sources. The detailed procedures for their extraction and fractionation are beyond the scope of this paper.

Apparatus

A diaphragm compressor (Nova, Effretikon, Switzerland) is used to fill a stainless-steel buffer tank with highly pressurized CO_2. The CO_2 is then decompressed to the working pressure by a pressure-reducing regulator (Tescom, Elk River, MN, U.S.A.). The CO_2 is mixed with the modifier (methanol) delivered by a syringe pump (MicroGradient System; Brownlee Labs., Applied Biosystems, Santa Clara, CA, U.S.A.), enters the oven and is directed to the six-port injection valve (AC6W; Valco Instruments, Houston, TX, U.S.A.). A direct and accurate determination of the proportion of modifier is not possible as the CO_2-delivery system works on pressure

control whereas the modifier pump works on flow control. However, the proportions of modifier were calculated to be *ca.* 2–3% in all instances. Detection is performed either with a UV detector (UVIS 20; Carlo Erba, Milan, Italy) or with a laser light-scattering detector (Varex, Rockville, MD, U.S.A.), or with both detectors simultaneously. With laser light-scattering detection (LLSD), the flow is split at the outlet of the column and 5–10% is directed to the drift tube of the detector. The remaining part (or the total if the LLSD instrument is not connected) of the flow passes through the high-pressure UV cell, and is directed either to a ten port valve (MCSD10UW; Valco Instruments) fitted with the collection vials or to the TLC interface. These various elements are interconnected with 1/16 in. O.D. and 0.5 mm I.D. stainless-steel tubing. The same tubing is pinched to produce the necessary restrictions in the collection system, the TLC interface and the LLSD instrument. The metallic tubes and glass vials used for collection are shown in Fig. 3 and elsewhere [6]. The TLC interface and plate conveyor have been described previously [7].

The separations were performed on C_{18} columns [laboratory-filled, 25 cm × 4.6 mm I.D., 10 μm; Supelco, (Bellefonte, PA, U.S.A.), 25 cm × 10 mm I.D., 5 μm; Supelco, 25 cm × 21 mm I.D., 15 μm] and silica gel TLC plates (20 × 20 cm × 2 mm) (No. 5717; Merck, Darmstadt, Germany). The flow-rates were typically 2 ml/min for the 4.6 mm I.D. column, 3.5 ml/min for the 10 mm I.D. column and 14 ml/min for the 21 mm I.D. column. Other chromatographic conditions are given in the legends of the figures.

Detection on TLC plates

General method. A saturated solution of berberine chloride (Fluka, Buchs, Switzerland) in ethanol is sprayed on the plate. The solutes give spots under UV irradiation (254 or 366 nm).

Specific detection. Immediately after elution of the preparative TLC plate, a TLC plastic sheet (20 × 20 cm × 0.2 mm) (No. 5735; Merck) is pressed strongly against the preparative plate for 1–2 min. Alternatively, the preparative plate can be dried and briefly dipped in ethanol before contact. Detection of the spots on the plastic sheet by any method [in this instance with 2,6-dichloroquinonechlorimide (Fluka) for phenol detection] gives the mirror image of the main TLC plate.

RESULTS AND DISCUSSION

Semi-preparative columns

Although the development of high-field Fourier transform (FT) NMR instruments has dramatically increased their sensitivity, NMR analyses still require relatively large amounts of samples, typically in the range 0.5–5 mg for ^{1}H NMR and 5–50 mg for ^{13}C NMR. Such amounts cannot be separated efficiently with the 4.6 mm I.D. columns, but fit well with the sample capacity of 10 and 21 mm I.D. packed columns. Only a few reports of SFC separations with 10 mm I.D. [11,12] and 20 mm I.D. [13,14] column have appeared. Larger-scale SFC separations have been carried out by Perrut and Jusforgues [15]. Both small- and large-scale preparative SFC have recently been reviewed by Berger and Perrut [16].

Fig. 1 compares the separation of tocopherol isomers with 4.6, 10 and 21 mm I.D. columns. Very similar resolutions are obtained, provided that the compression

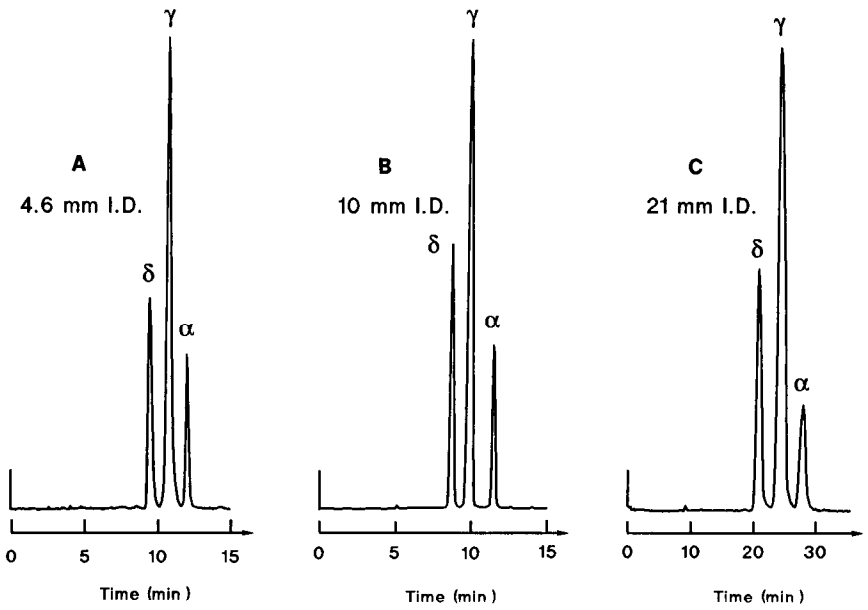

Fig. 1. Separation of a mixture of α-, γ- and δ-tocopherols on C_{18} packed columns. (A) Injection of 0.6 mg on the 4.6 mm I.D. column; (B) injection of 2.5 mg on the 10 mm I.D. column; (C) injection of 10 mg on the 21 mm I.D. column. Eluent: CO_2 + methanol (see Experimental). Temperature, 40°C; pressure, 240 bar, UV detection at 290 nm.

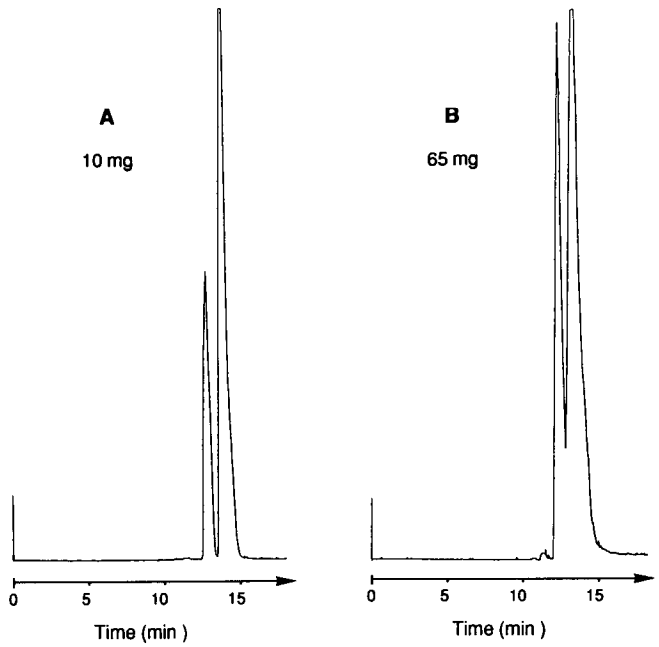

Fig. 2. Separation of phytol isomers with the 21 mm I.D. column. Injection of (A) 10 mg and (B) 65 mg. Eluent: CO_2 + methanol (see Experimental). Temperature, 40°C; pressure, 250 bar; LLSD detection.

system can deliver enough mobile phase and that the pressure drop is kept reasonably low by the use of 0.5 mm I.D. tubing. The high capacity of semipreparative columns is demonstrated in Fig. 2. With the 21 mm I.D. column, complete separation of up to 10 mg of *cis–trans*-phytol can be achieved, and the separation of 65 mg of the same mixture without significant loss of resolution is possible. In this example, the amounts of separated compounds are in the range of ^{13}C NMR requirements.

Collection systems

Obviously, semi-preparative separations are performed with the aim of isolating a maximum amount of pure compounds, so the efficiency of the collection step is of prime importance, For SFC with CO_2, the decompression of the supercritical eluent to atmospheric pressure at the terminal restriction allows its straightforward removal, but also induces a very high flow-rate of gas, which can blow the compounds out of the collection vials. Different systems of collection exist and can be divided in two groups. One involves collection of the solutes before complete decompression, and therefore requires pressure-resistant collection devices [17,18]. Alternatively, the eluent can be decompressed to atmospheric pressure and the solutes trapped from the resulting gas flow [11,12,19,20]. The latter method is usually more convenient but less efficient [11]. Some collectors are also incompatible with the high flow-rates of semi-preparative columns [20].

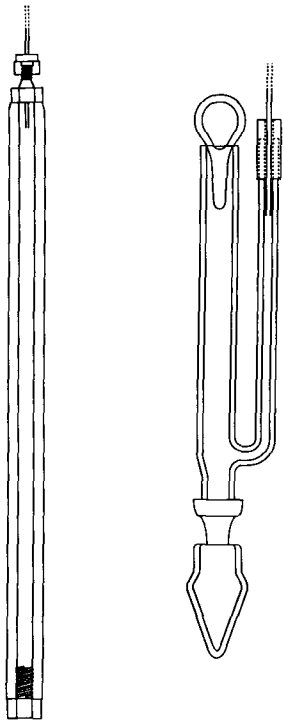

Fig. 3. Collection vials. (A) Stainless-steel tube for collection under pressure; (B) glass vials for collection after decompression.

We used three simple systems of collection and compared their efficiencies. The first is similar to the system described by Campbell and Lee [18]. It consists of closed stainless-steel tubes, into which the fractions are collected under pressure, *i.e.*, in the liquid state (Fig. 3A). The tubes are then very gently decompressed to remove the CO_2. The second type of collector consists of specially shaped glass vials, which trap the dry-ice–modifier droplets produced by the decompression of the mobile phase [6]. (Fig. 3B). A heating system prevents the formation of a blockage of dry ice in the lateral tube. In the third method, the outlet of the restrictor is directed toward a TLC plate, which is slowly moved on a conveyor [7]. After elution, the compounds are recovered by stripping the adsorbent from the plate and washing the silica with a small volume of organic solvent.

Table I compares the recoveries of isophytol and benzyl benzoate after separation and collection with each system. In fact, these values also include losses due to other parts of the chromatographic system, which represent about 10% of the injected amounts. After correction, the efficiences of the collectors alone are above 90%, except for the TLC system.

Although these results show high efficiencies for both the stainless-steel tube and glass systems, we strongly favour the latter owing to its simplicity and convenience of use. In comparison, the deposition of the eluent on a TLC plate leads to significant losses.

We carried out numerous experiments in order to understand which parameters govern the efficiency of the SFC to TLC transfer. As a result, the yield of deposition was found to be independent of the nature of the solute (functional groups, polarity), its molecular weight (for mol. wt. > 300), and the amount of compound (in the range 1–10 mg). Further, deposition on TLC plates with and without a concentration zone leads to similar results.

On the other hand, the yield of deposition is strongly dependent on the presence of the modifier, the shape of the jet and its temperature, which varies with the flow-rate and the power applied to the restrictor heating block.

Moreover, these parameters interdependently affect the transfer; the best yields are achieved when all the mentioned parameters are set such that the jet of CO_2 leaves a sharp wet line of modifier on the TLC plate. If this condition is not fulfilled, owing to absence of modifier or an unsuitable temperature, poorer transfer occurs, even when the TLC plate is pre-wetted with solvent prior to deposition.

Substrate losses result from the difficulty of retaining on the silica plate the effluent particles expelled at high speed from the restrictor. Obviously, liquid droplets

TABLE I

YIELD OF RECOVERY OF THE COLLECTION SYSTEMS

SFC: 10 mm I.D. column; eluent, CO_2 + methanol (see Experimental). Mean results of three experiments; recovered amounts determined by GC with triplicate on-column injections.

Collection system	Benzyl benzoate (%)	Isophytol (%)
Stainless-steel tube	86	89
Glass vial	85	82
TLC plate	–	36

are held better than solid or gaseous material. Hence, the transfer is enhanced by allowing the solute to be trapped in droplets of modifier and deposited as such on the TLC plate.

On-line coupling of semi-preparative SFC with semi-preparative TLC

Despite its low efficiency, collection of SFC effluents on TLC plates presents obvious advantages. After development, the thin-layer plate can be sprayed with detection reagents, offering a means of detection and a purity check of the SFC fractions [7]. Alternatively, the plate may be used for FT-IR spectroscopy [10,21].

However, the major benefit of this coupling relies on its capability to produce a two-dimensional semi-preparative separation. Semi-preparative TLC is a cheap and widespread method for the separation of non-volatile compounds. One can purchase or prepare TLC plates coated with numerous types of adsorbents; the choice covers normal phases, reversed phases, chiral phases and impregnated supports (*e.g.*, silver nitrate) [22]. However, semi-preparative TLC has a poor resolving power, which often precludes the isolation of pure compounds. Finally, the typical adsorbent thick-

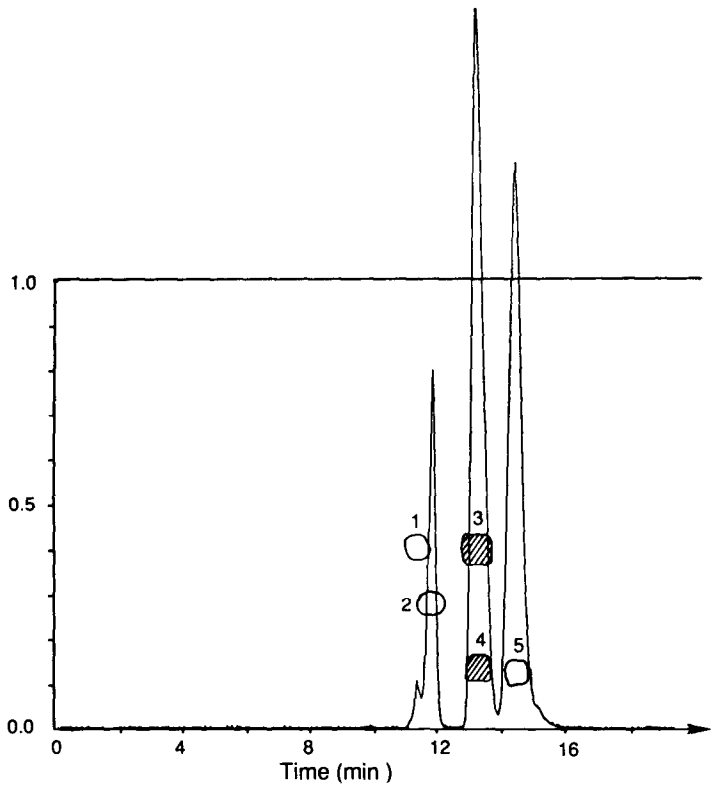

Fig. 4. Two-dimensional SFC–TLC separation. SFC: 21 mm I.D. column; eluent, CO_2 + methanol (see Experimental); temperature, 40°C; pressure, 210 bar; LLSD detection. TLC: solvent, hexane–ethyl acetate (9:1); detection reagent, berberine chloride; UV detection. 1 = Methyl palmitate; 2 = isophytol; 3 = methyl stearate; 4 = *cis*-phytol; 5 = *trans*-phytol. The NMR spectra of the products corresponding to the shaded spots were recorded.

ness (2 mm) allows the deposition of about 7 mg/cm [23], fitting perfectly with the capacity of semi-preparative SFC columns. As a result, semi-preparative TLC emerges as a natural complement to SFC for separations of milligram amounts of complex mixtures.

The two-dimensional separation results in spots of solutes spread all over the TLC plate. For collection, they must be accurately located using a non-destructive method. Two alternatives are possible, depending on whether the detection process should be non-specific or should detect only one class of compounds.

The general detection method takes advantage of berberine chloride. This reagent, when sprayed on the plate, makes organic compounds UV detectable, even fully saturated hydrocarbons. It is insoluble in most of the usual solvents for TLC and remains on normal-phase silica when the compounds are recovered by washing. Surprisingly, we have found only one publication referring to berberine chloride as a detection reagent [24], although it is routinely used in some laboratories [25]. As an example, fig. 4 shows the separation of a mixture of five compounds. Neither SFC nor TLC alone would have led to a complete separation of this sample. In Fig. 5, the same technique was applied to a natural fraction of jasmine extract. In both experiments, the shaded sports were stripped from the plate and the silica was washed with diethyl ether to afford about 1 mg of pure compounds. This was sufficient for both ^{1}H NMR and mass spectrometry and to identify the compounds isolated from jasmine extract as isophytol, phytyl acetate, phytol, squalene epoxide and phytyl palmitate.

We developed a second method of detection to be used whenever the berberine chloride method cannot be applied or if a specific class of substances is under in-

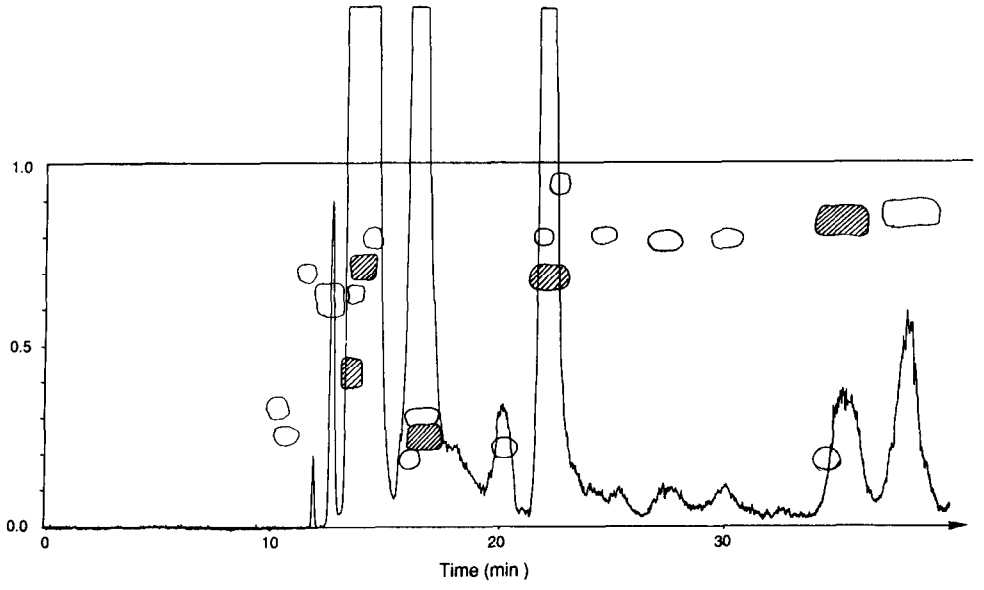

Fig. 5. Two-dimensional SFC–TLC of a fraction of jasmine extract. SFC: 21 mm I.D. column; eluent, CO_2 + methanol (see Experimental); temperature, 40°C; pressure, 300 bar; LLSD detection. TLC: solvent, hexane–diethyl ether (4:1); detection reagent, berberine chloride; UV detection. The NMR spectra of the products corresponding to the shaded spots were recorded.

vestigation. By firmly pressing a soft (plastic) TLC sheet against the wet, thick plate, tiny fractions of the compounds are transferred. The plastic plate can then be treated by any method, giving the mirror image of the spots of interest. A similar method using filter-paper was mentioned by Mitsryukov [26]. In Fig. 6, we used 2,6-dichloroquinonechlorimide, a specific reagent for phenols. Thus, it was possible to localize selectively tocopherols in a fraction of coffee extract. In this instance, the main plate was also sprayed with berberine chloride, showing that all the compounds in this fraction have similar R_F values on TLC. The mass spectrum of the stripped compound indicates β-tocopherol or γ-tocopherol, but it is not informative enough to distinguish between them. The recovery of sufficient material for NMR analysis permitted the identification of β-tocopherol.

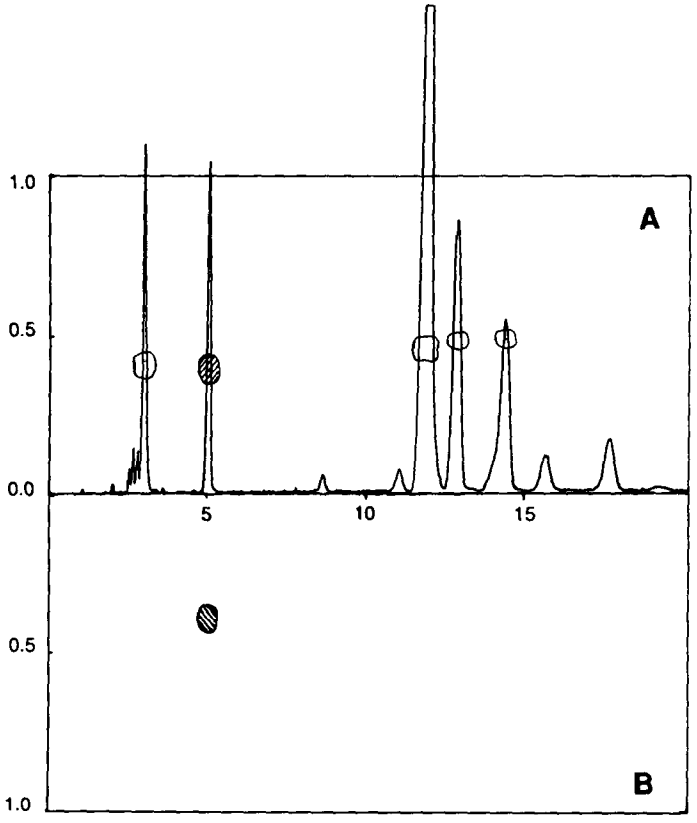

Fig. 6. Two-dimensional SFC–TLC of a fraction of coffee extract. SFC: 10 mm I.D. column; eluent, CO_2 + methanol (see Experimental); temperature, 40°C; pressure, 250 bar; LLSD detection. TLC: solvent, hexane–ethyl acetate (9:1); detection reagent, (A) berberine chloride and (B) 2,6-dichloroquinonechlorimide; UV detection. The NMR spectra of the products corresponding to the shaded spots were recorded.

CONCLUSIONS

Although most of the publications devoted to SFC concern analyses performed with open-tubular or packed capillary columns, SFC also offers very attractive possibilities for larger scale separations. In the semi-preparative mode, the benefits of SFC over GC, *i.e.*, low-temperature separation of the heat-sensitive or non-volatile compounds, are still valid. Further, the benefits of SFC over HPLC, *i.e.*, speed of analysis, non-toxicity and a readily removed mobile phase, are amplified on the semi-preparative scale in comparison with the analytical scale.

Semi-preparative SFC utilizes large-diameter HPLC packed columns filled with very fine and homogeneous silica particles and therefore takes advantage of their unequalled efficiency for separations of relatively large amounts of material. We were able to isolate tens of milligrams of compounds in one injection thanks to the use of such columns and to the development of convenient and efficient collection system.

Alternatively, the effluent can be diverted to a semi-preparative TLC plate for further purification, leading to a unique two-dimensional semi-preparative separation.

However, significant losses occur during the direct transfer from SFC to TLC. If the sample is available in limited amount or if the compounds of interest are minor constituents, fractions should be collected and subsequently deposited on the plate. A non-destructive method must be used to detect the spots. A normal-phase support can be treated with berberine chloride reagent. A second detection method was tested for other adsorbents or for specific detection.

Both high-performance packed columns and TLC plates are available with numerous types of coating. Their combination offers a broad range of selectivity and covers a large field of applications. It turns out to be a particularly useful method when milligram amounts of pure substances have to be isolated from complex mixtures.

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