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Preparative-scale supercritical fluid chromatography

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Abstract

An apparatus for the study of preparative supercritical fluid chromatography (SFC) has been built and used to study the separation of a model system [a 1:1 (w/w) mixture of fluorene and phenanthrene] and a more real system (a mixture of milbemycins containing also other extracted material). The principles of preparative SFC are given and applied to these studies, and some conclusions about the optimisation of preparative SFC drawn.

1. Introduction

Because solubility and diffusion can be optimised by controlling both pressure and temperature, chromatography using a supercritical fluid as the mobile phase (SFC) can achieve better and more rapid separations than liquid chromatography. It is now being used preparatively to separate high-value materials, such as pharmaceuticals, where preparative high-performance liquid chromatography (HPLC) is difficult. Because peaks are narrower in SFC, and for these difficult separations very little overloading can be done so that advantage can be taken of the narrow peaks obtained, the maximum amount of material obtained in a run is of the order of 100 mg in SFC compared with the 1-g amounts obtainable sometimes in HPLC. The equipment described in the next section was built in the laboratory, but commercial systems on a preparative and larger scale are being developed. A number of reports [1–11] of preparative SFC have appeared. The studies described here are aimed at understanding the principles and optimisation of preparative SFC.

2. Experimental

Fig. 1 shows the preparative system, which consists of a pumping system; a core chromatographic section, containing an injection loop, column and detector; and a trapping system followed by a back-pressure regulator to maintain the pressure of the whole system.

2.1. Pumping system

The pumping system is capable of delivering a mixed fluid (e.g. carbon dioxide modified with

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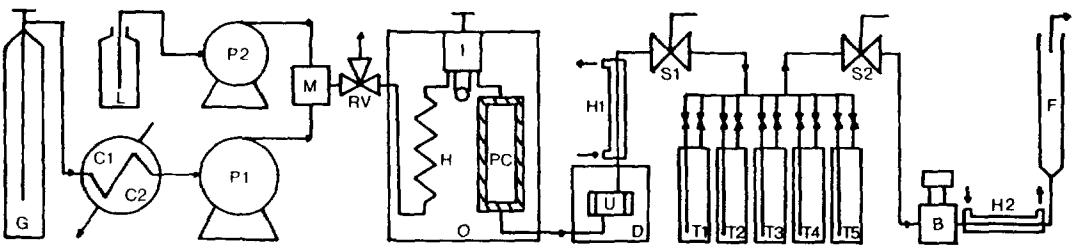

Fig. 1. Preparative SFC system. G = CO_2 cylinder; C1, C2 = condensers; P1 = dual-head reciprocating pump; P2 = liquid pump; M = dynamic mixer and damper; RV = pressure-release valve; O = oven; H = preheater; I = injection valve; PC = preparative column; D = UV detector; U = UV cell; H1 = heat exchanger for cooling; S1, S2 = 7-port valves for switching between traps; T1–T5 = traps; B = back-pressure regulator; H2 = heat exchanger; F = bubble flow meter.

methanol) at a flow-rate of 25 ml CO_2/min and 5 ml/min of modifier at a pressure of 300 bar. Such a system requires judicious choice of pump and efficient cooling of CO_2 before entering the pump and in the pump head to ensure that the CO_2 is pumped as a liquid. CO_2, supplied from a cylinder with a siphon tube, is passed a Nupro filter and cooled in two condensers, C1 and C2. C1 is a 75-ml sample cylinder (Whitey, Manchester Valve and Fitting Co.) fitted inside a coiled copper tube and insulated with polystyrene foam. C2 is constructed from a 5 m length of stainless-steel tubing (6 mm nominal O.D. and 2.16 mm I.D.), which is coiled and placed inside a copper jacket. The CO_2 pump, P1, is a dual-head reciprocating type (Varex, P.S. Instruments) as is the modifier pump, P2 (Gilson). The condensers, C1 and C2, and the CO_2 pump head are cooled by a refrigerated circulating bath (Julabo, Jencons Scientific) to −4°C. A dynamic mixer and damper (Gilson) are used to provide the even flow and composition needed for efficient SFC. A pressure-relief valve, RV, is placed in the line between the pumping system and chromatographic section, set to 300 bar, to which a Bourdon pressure gauge, 0–400 bar, is connected (Rheodyne).

2.2. Chromatographic section

After passing through a preheater, H, consisting of a 5 m length of stainless-steel tubing of 1.5 mm O.D. and 0.5 mm I.D., to bring it to supercritical conditions, the fluid then passes through an injection valve, I, (Rheodyne) with a 1-ml sample loop. A preparative column designed for HPLC, 250 mm × 20 mm I.D. (Phase Separations) is enclosed in a pressure-safety vessel, made in the laboratory. The preheater, injection valve and column are installed in an HPLC oven, O (Dupont), controllable between ambient and 100°C. The temperature of the oven is measured using a type K thermocouple connected to a digital display unit (Ametek). Temperature stability of the oven is ± 0.5°C. Pressures at the inlet and outlet of the column are measured using pressure transducers and control units (Thorn-EMI Datatech, Model SE42 and Lintronic, Model Setra 205-2), not shown. Pressure drops of 10–60 bar across the column and the data referred to the average column pressure. A UV detector, D, containing a high-pressure front-loading cassette cell, U, of 4 μl volume and 5 mm path length (Jasco, Model 875), is used to follow the separation.

2.3. Trapping system

After leaving the chromatographic section, the eluent is cooled in a coil in a water bath to ambient temperature. Trapping of fractions of the peaks is thus done at room temperature where the mobile phase is liquid. The five traps, T1–T5, made in the laboratory, are 60 ml in volume and are connected sequentially into the flow via the 7-port valves, S1 and S2 (Rheodyne 7060) at times indicated by the chromatogram received by the detector. Of the seven ports on each of these valves, five connect via each of the traps, one connects the two valves directly together for use before and after collection, and one goes to vent. The traps are each fitted with

two isolating valves (SSI, Scientific Glass Engineering). The traps are filled with the liquid CO_2 before the separation is carried out and during collection not more than 50% of liquid CO_2 is displaced. At the end of the separation, the traps are frozen in liquid nitrogen and opened to the atmosphere, when the mobile phase slowly evaporates. This type of trapping system is not necessarily the most suitable for routine preparative SFC, but gives quantitative collection and is suitable for the studies described in this paper. After the solid CO_2 has evaporated, the solute is then collected from the traps using an organic solvent and analysed. The back-pressure regulator, B, heated mechanical (GO, Model BP-66) or electromechanical (Jasco, Model 880-81) maintains the required pressure in the system and a bubble flow meter is installed, to check the flow-rates indicated on the pumps, following a simple heat-exchanger to bring the emerging gas up to room temperature, H2.

3. Principles of preparative SFC

For a discussion of principles, a 1:1 (w/w) mixture of two compounds is considered. If their two peaks are well separated, a plot of purity versus the fraction of total material collected will appear as curve A in Fig. 2. The first half

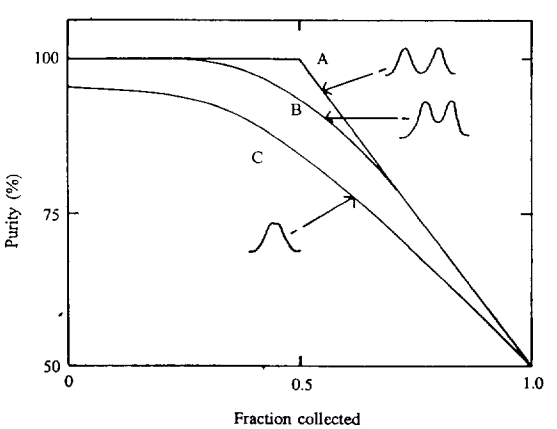

Fig. 2. Schematic plot of purity versus fraction collected for qualities of separation decreasing A to B to C. See text.

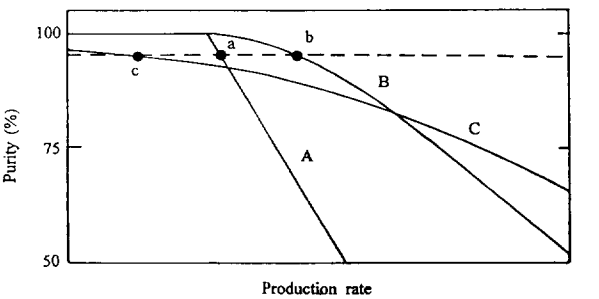

Fig. 3. Schematic plot of purity versus production rate for various qualities of separation decreasing A to B to C, due to increasing loading or flow-rate. See text.

collected will be one pure compound, which will progressively contaminated by the second compound as it is added in to the material collected. If the separation is worse, curve B will be obtained, with the second compound emerging before all the first compound has been collected. If separation is poor, curve C is obtained, with some of the second compound emerging initially.

For a given separation under a given set of other conditions, worse separation is obtained if either the flow-rate or loading is increased. At the same time, however, the production rate is increased. Thus, if purity is plotted against production rate, the curves A, B and C of Fig. 2 will become spread out as in Fig. 3. This is the form in which data are commonly presented, for example in the next section. The horizontal dashed line in Fig. 3 represents a purity of 90% and it cuts the curves A, B and C at the points a, b and c. These points are also shown on Fig. 4 as a schematic plot of production rate versus loading or flow-rate for a purity of 90%. Fig. 4 thus

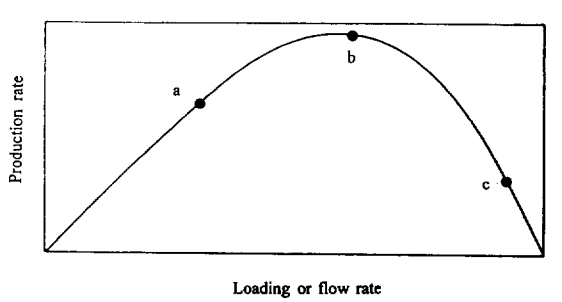

Fig. 4. Schematic plot of production rate versus loading or flow-rate for a given required purity. See text.

shows how an optimum loading or flow-rate arises for a given required purity.

4. Studies using the experimental system

Studies were carried out initially on a 1:1 (w/w) mixture of phenanthrene and fluorene, chosen because they elute close together in SFC. Phenanthrene (BDH) and fluorene (Aldrich) were checked for purity using GC and found to be 98.8 and 97% pure, respectively. After the traps were filled and the system had reached equilibrium, 1 ml of solution of various loadings was injected. Chromatography was carried out using a preparative column designed for HPLC, 250 mm × 20 mm I.D., packed with 10-μm diameter silica particles bonded with octadecylsilyl groups (Phase Separations, type ODS2). In the experiments described here, chromatography was carried out at 40°C and 250 bar. Pure CO_2 (BOC 99.98% purity) and CO_2 modified with up to 20% methanol (v/v at the pumps) was used at CO_2 flow-rates up to 25 ml/min and at loadings of up to 250 mg of the mixture. Removal of the fractions from the traps was done using dichloromethane (FSA Labs., 99.8% quoted purity) and analysis of the five fractions collected was done by UV absorption at 295 and 302 nm for phenanthrene and fluorene, respectively. In a small number of cases, where the concentrations were too small for UV analysis, GC determinations were made. After analysis of the fractions, results from individual fractions and theoretical combinations of the fractions enabled plots of purity versus production rate to be drawn. Such plots for fluorene recovery (the second peak eluted) are shown both for increasing loading in Fig. 5 and increasing flow-rate in Fig. 6. The plots are seen to be of the form of Fig. 4 for both loading and flow-rate.

Studies were then carried out on a more real system; the separation of milbemycin α_2 from a toluene extract of microbial cells. This extract contained only ca. 2.5% of milbemycin α_2 (eluted first), other milbemycins and other compounds. The maximum production of milbemycin α_2 of 90% purity obtained in a single

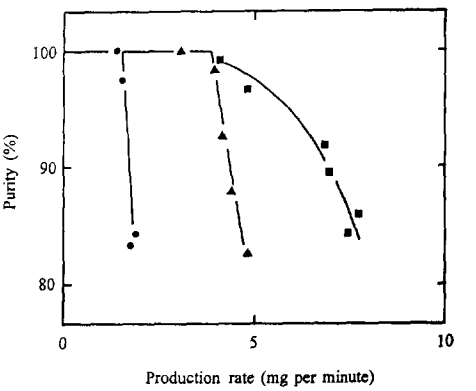

Fig. 5. Purity versus production rate of fluorene for the preparative SFC of phenanthrene–fluorene (1:1, w/w) at loadings of: ● = 30 mg; ▲ = 75 mg; ■ = 108 mg. Column, 250 mm × 20 mm I.D. containing octadecylsilyl-bonded 10-μm silica particles; temperature, 40°C; pressure, 250 bar; mobile phase, pure CO_2; flow-rate of CO_2, 20 ml/min.

run was 10 mg. Initial conditions were sought on an analytical scale, using the same system with analytical columns, 250 mm × 4.6 mm I.D. (Phase Separations). Best separation was ob-

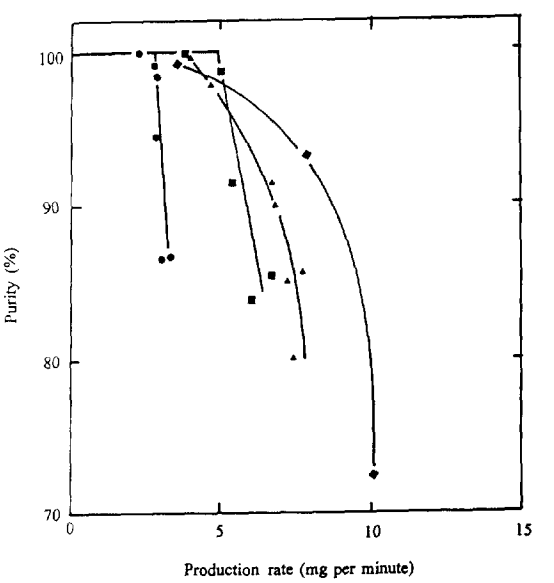

Fig. 6. Purity versus production rate of fluorene for the preparative SFC of phenanthrene–fluorene (1:1, w/w) at flow-rates of: ● = 10 ml/min; ■ = 15 ml/min; ▲ = 20 ml/min; ◆ = 25 ml/min. Column, 250 mm × 20 mm I.D. containing octadecylsilyl-bonded 10-μm silica particles; temperature, 40°C; pressure, 250 bar; mobile phase, pure CO_2; loading 108 mg.

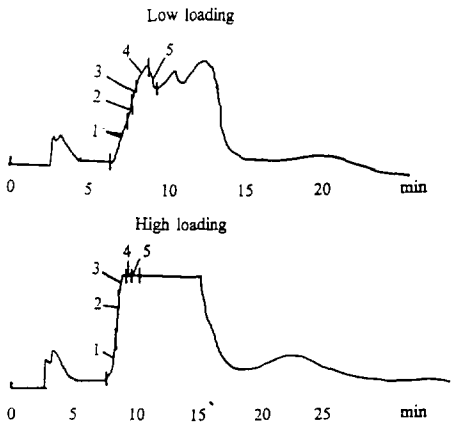

Fig. 7. Chromatograms obtained from the detector of the preparative SFC system for the separation of milbemycin α_2 at low (0.1 g) and high (0.5 g) loadings of crude extract. Column, 250 mm × 20 mm I.D. containing cyanopropyl-bonded 5-μm silica particles; temperature, 47°C; pressure, 120 bar; mobile phase, 5% methanol in CO_2 (v/v at pumps); flow-rate of CO_2, 20 ml/min.

tained using a column packed with cyanopropyl bonded 5-μm silica particles, a temperature of 47°C and a pressure of 120 bar using a mobile phase of 5% methanol in CO_2 (v/v at the pumps). Preparative chromatography was then carried out on a column of 250 mm × 20 mm I.D. containing the same packing (Phase Separations) and under the same conditions. Analysis of samples was carried out by HPLC. Examples of chromatograms obtained from the detector of the preparative SFC system at low (0.1 g) and high (0.5 g) loadings of crude extract, using a 20 ml/min flow-rate of CO_2 are given in Fig. 7. This figure also shows the fractions collected, from which curves of purity versus production rate were obtained and shown in Fig. 8.

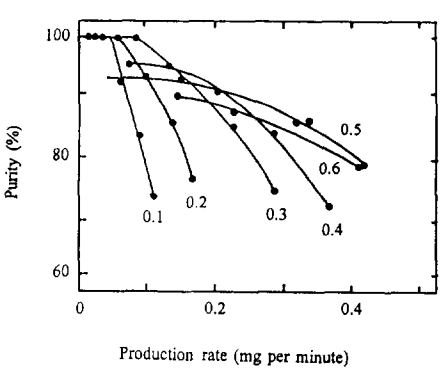

Fig. 8. Purity versus production rate for the preparative SFC of milbemycin α_2 at various loadings of crude extract, shown in grams on the figure. Other chromatographic conditions as in Fig. 7.

5. Optimising preparative SFC

The parameters to be optimised during preparative SFC are column type, temperature, pressure, modifier concentration, flow-rate and loading. The initial search for conditions is carried out by observing the chromatogram obtained, without trapping. It is more economical if at least some of this work is carried out on an analytical-scale SFC, particularly column, pressure, temperature and modifier concentration, as in the milbemycin study, described above. The initial aim of this study was to look for methods of optimising all parameters, but the experience obtained with the experiments described and others is that further optimisation of column type, pressure and temperature is not worthwhile and that further optimisation of modifier concentration (discussed further below) is of questionable worth. However, for further refinement of flow-rate and loading conditions, trapping experiments as described in the last section can be carried out to obtain curves of purity versus production rate for different conditions. These curves can then be further analysed, as is now described for the effect of loading on the isolation of milbemycin α_2. Below 100% purity, the curves of Fig. 8 are fitted to a quadratic and the equations obtained are used to calculate values of the production rate at a given purity for the various loadings. Curves can then be drawn of production rate versus loading for a given purity and this is shown in Fig. 9 for the milbemycin data. As can be seen, there is an optimum loading, for a certain required purity. Similar curves can be obtained for the effect of flow-rate.

Theoretically, a two-dimensional optimisation for both loading and flow-rate could be carried out, and although we have had some success in this, errors in the experimental data make this

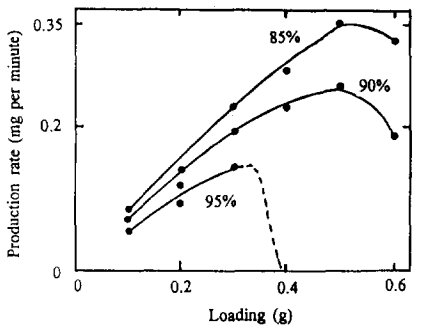

Fig. 9. Production rate versus purity for the preparative SFC of milbemycin α_2 for various required purities, shown on the figure. Column, temperature, pressure and flow-rate as in Fig. 7. A purity of 95% was not obtainable at loadings of 0.4 g and above and so a schematic dashed line is shown.

difficult and the results are not worth reporting. It appears to be better to choose an optimum flow-rate from observation of the chromatograms, obtain an optimum loading by the procedure outlined above and then refine flow-rate by the same procedure. Further iterative optimisations are possible.

As far as modifier concentration is concerned, optimisation by observation of the chromatograph is probably the only viable method, but more quantitative optimisation may be possible for some systems. Good separation of milbemycin α_2 could not be obtained with pure CO_2, improved significantly with 5% added methanol, but did not improve further with greater addition of methanol. For the phenanthrene–fluorene system, quantitative optimisation of modifier concentration was attempted as, although modifier is not needed to affect the separation, it is known that added modifier reduces the tailing of peaks in SFC caused by unbonded sites on the packing and therefore might improve the purities achieved. Experiments were carried out at high loadings and flow-rates. However, as seen in Fig. 10, the effect of modifier, if any, is less than the experimental scatter, which is greater than previously because of more rapid elution, and no improved separation is achieved.

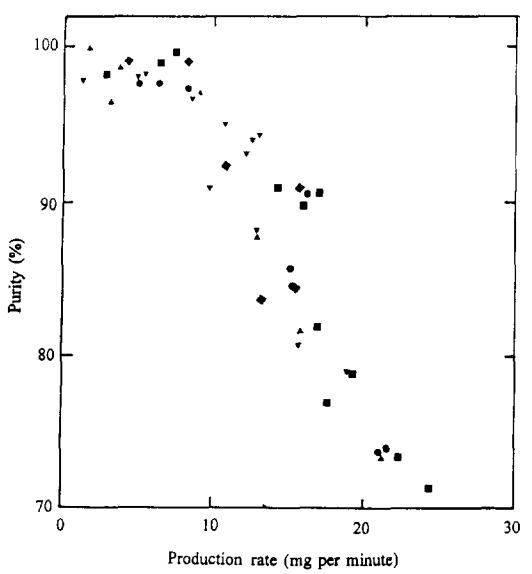

Fig. 10. Purity versus production rate of fluorene for the preparative SFC of phenanthrene–fluorene (1:1, w/w) using CO_2 containing methanol at: ● = 0%; ◆ = 2%; ▼ = 5%; ■ = 10%; ▲ = 20% (v/v). Column, 250 mm × 20 mm I.D. containing octadecylsilyl-bonded 10-μm silica particles; temperature, 40°C; pressure, 250 bar; mobile phase modified CO_2; flow-rate of CO_2, 20 ml/min; loading 250 mg.

6. Conclusions

It is concluded that of the parameters that can be varied in preparative SFC, those of column type, pressure and temperature probably modifier concentration are best chosen by observations of separation on the chromatograms obtained. Further, more quantitative, optimisation can be carried out on flow-rate and loading separately, and perhaps iteratively, by a procedure described above. This procedure involves carrying out trapping experiments at different conditions of loading or flow-rate, plotting curves of purity versus production rate, and fitting these curves to obtain plots of production rate versus loading or flow-rate to obtain optima for a given desired purity. Optimisation along the lines suggested is worthwhile for a preparative SFC that is likely to carried out often and especially for a system which may be taken up to production scale.

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