Journal of Chromatography, 605 (1992) 95–101
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 179

Packed column subcritical fluid chromatography of underivatized amino acids

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(First received November 25th, 1991; revised manuscript received March 13th, 1992)

ABSTRACT

Packed column subcritical fluid chromatography of underivatized amino acids is described. Using pyridine (or ethylene glycol)–methanol–water–triethylamine as modifier in the carbon dioxide, mixtures of amino acids can be separated on diol-bonded silica and detected without derivatization using evaporative light-scattering detection. Pyridine and ethylene glycol are shown to impregnate the stationary phase and to improve efficiency. The results demonstrate the wide potential of packed column subcritical fluid chromatography for the determination of polar compounds.

INTRODUCTION

Many different methods have been used to separate amino acids by liquid chromatography (LC); in most instances, precolumn or postcolumn derivatization is required to allow UV absorptiometry or fluorescence detection and to mask reactive functions. Thus, the use of reagents such as *o*-phthaldialdehyde (OPA) [1–3], 9-fluorenylmethylchloroformate (FMOC) [4] and phenyl isothiocyanate (PITC) to form phenylthiohydantoin (PTH) derivatives [5] has been extensively investigated and apparatus involving these techniques has been automated.

In contrast, to our knowledge, only four references are devoted to supercritical fluid chromatography (SFC) of amino acids [6–9]. They involve precolumn derivatization with PITC or FMOC on bare silica [6,9] or on cyanopropyl-bonded silica [7,8] and separations require a high modifier content in the carbon dioxide to dissolve amino acids in combination with an elution gradient. Derivatization was used to advantage for detection and mainly to reduce the polarity of amino acids by masking their polar functions. Employing a high percentage of a modifier means that the mobile phase was subcritical instead of supercritical. Thus, under these conditions, the kinetic advantages of subcritical fluid chromatography (SubFC) are reduced owing to the lower diffusion coefficients.

The SubFC separation and detection of amino acids without derivatization were a challenge: clearly the polarity of CO_2 is too low to dissolve amino acids without adding a high concentration of modifiers. The mobile phase has to be strong enough to elute them from a packed column while keeping the selectivity high enough to obtain a good resolution. This requires the stationary phase to be chosen with care in order to retain and elute the amino acids: with a low-polarity stationary phase, they would

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0021-9673/92/$05.00

elute without retention; with a polar stationary phase, amino acids would have too strong interactions to permit elution under reasonable conditions, *i.e.*, with a modifier content in the CO_2 lower than 30%. After selecting a suitable stationary phase, the mobile phase must control the retention and solubility of the solutes. On packed columns, this can be done by adding polar modifiers to the CO_2 and varying their contents or, for small amounts of modifiers, by varying the CO_2 density [10]. Hence very polar modifiers have been used to separate polyhydroxybenzoic acids and benzenepolycarboxylic acids [11,12], phenols [13], imidazole derivatives [14], opium alkaloids [15] and various chiral species such as β-blocking drugs [16,17]. It has been demonstrated that polar modifiers improve peak shapes and promote the elution of polar compounds by clustering together: the solvent strength in the clusters where the solutes are localized is much higher than that in the bulk mobile phase [12]. Hence it is necessary to add modifiers to the mobile phase, but selecting the modifiers is not trivial with regard to amino acids.

From the detection point of view, the challenge was solved using the experience gained in our laboratory with evaporative light-scattering detection (ELSD) coupled to SubFC [18–21] or, in some experiments, by adding copper ions to the polar modifier to obtain complexes of amino acids giving suitable UV absorption at 254 nm [22].

It should be borne in mind that this work was undertaken mainly to show the extreme limits of packed column CO_2 SubFC for the separation of very polar compounds, and not to compete with LC.

EXPERIMENTAL

SubFC with UV detection

A newly developed prototype of supercritical fluid chromatograph (Gilson Villiers-le-Bel, France) was coupled to a Varian (Orsay, France) Model 2550 UV detector. It consists of a Model 305 piston pump (main pump) with its head cooled to 0°C to deliver the CO_2, a Model 306 piston pump (slave pump) for the modifiers, a Model 811 B mixing chamber (two-stage dynamic mixing, 1.5 ml total volume) connected to the pulse damper module, a Rheodyne injection valve equipped with a 20-μl loop, a column oven and a manometric module combined with a pulse damper and a first-stage back-pressure regulator. This new module provides column inlet and outlet pressures to the main pump. The outlet pressure can be monitored using the main pump software, allowing pressure gradients in combination with modifier gradients by increasing the modifier flow-rate. A Tescom back-pressure regulator set at 80 bar ensured final expansion of the mobile phase. The chromatograms were stored in a Shimadzu Model C-R6A integrator (Touzart et Matignon, Vitry-sur-Seine, France).

SubFC with evaporative light-scattering detection (ELSD)

A liquid chromatograph modified for packed column SFC (Varian Model 2500 M) was used without modification. It consists of two Model 2510 piston pumps that allow programming of composition gradients, a dynamic mixing chamber (Gilson, Model 811B) and a Valco injection valve equipped with a 20-μl loop. The column was placed in a heated bath and connected to the interface of the ELSD (Sédex 45, Sédéré, Alfortville, France) designed for SFC. This interface replaces the LC interface and allows both expansion and nebulization of the mobile phase prior to detection using a calibrated fused-silica restrictor housed in stainless steel. In contrast to the LC device, the glass part connecting the interface to the evaporation tubing (set at 45°C) is thermoregulated at 70°C to avoid freezing. As usual [18,19], air supplies the SFC interface (pressure 1 bar) and the detection cell (pressure 2 bar) to improve nebulization and vaporization of the mobile phase and to reduce band broadening in the detection cell [18,19]. A Kipp & Zonen Model BD 40 plotter (Touzart et Matignon) was used to record the chromatograms.

Columns

The columns used for these experiments (Table I) were (1) 50, (2) 150 or (3) 250 × 4.6 mm I.D.

Solvents and solutes

CO_2 (industrial quality) was purchased from l'Air Liquide (Paris, France). Methanol and triethylamine were of high-performance liquid chromatographic grade from Prolabo (Paris, France). Pyridine, formic, citric and trifluoroacetic acid, eth-

TABLE I

STATIONARY PHASES USED FOR SubFC OF AMINO ACIDS

Column dimensions: (1) 50 × 4.6 mm I.D.; (2) 150 × 4.6 mm I.D.; (3) 250 × 4.6 mm I.D.

Stationary phase	Column	Diameter of particles (μm)	Company
Ultrabase UB 225 (C18)	3	5	SFCC Shandon, Eragny, France
Intersphère 1000 Å	3	7	Interchim, Montluçon, France
Intersphère-NH_2	2	5	Interchim
Nucleosil-NH_2	2	5	SFCC Shandon
LiChrospher 100 CN	2	5	Merck, Paris, France
Hypersil Deltabond CN	2	5	Touzart et Matignon
LiChrosorb diol	2	5	Interchim
LiChrosorb diol	1	5	SFCC Shandon

ylene glycol, glycerol, 2-propanol and propylamine were of analytical-reagent grade from Prolabo and Merck.

Amino acids were purchased from Prolabo and Merck. Samples to be injected were prepared by dissolving the amino acids in water–methanol (90:10, v/v) and then diluting them in the modifier to obtain 10^{-2} *M* solutions.

RESULTS AND DISCUSSION

Using the results obtained in the laboratory for the separation of alkaloids, imidazole derivatives and chiral species [14,15], we selected methanol–water–aliphatic amine as the main modifier to begin the experiments. In this slightly basic medium, amino acids were in the anionic form. To deal with positively charged amino acids, some experiments were also carried out with trifluoroacetic acid, citric acid or formic acid instead of triethylamine. The retention of amino acids on non-polar and polar stationary phases was first investigated to obtain rapid screening.

Preliminary experiments

Non-polar stationary phase. Experiments were carried out on Ultrabase end-capped octadecyl-bonded silica. Using 5–20% of methanol–water–triethylamine (or trifluoroacetic acid) (95.2:4.75:0.05, v/v/v) in CO_2, amino acids were not retained.

Polar stationary phases. Bare silica and amino-propyl-, nitrile- and diol-bonded phases were studied using similar mobile phases to those used with non-polar phases.

For a modifier content ranging from 10 to 20% in CO_2, the capacity factors of the amino acids vary (according to their polarity) from 0.5 to more than 30. Nevertheless, the efficiency is too low [less than 400 theoretical plates (TP)] to resolve mixtures of amino acids (elution peaks exhibit strong tailing).

Using acid modifiers, retention on the amino-bonded phase is too high to elute proline, used as a medium polarity test amino acid. In contrast, retention on Deltabond CN is very low and no separation of amino acids can be expected with this type of stationary phase and mobile phase. The best selectivity is obtained on LiChrospher CN and LiChrosorb diol-bonded silica but, unfortunately, the efficiency is still very low (less than 1000 TP).

Using a basic modifier, the results are almost the same: again, the greatest selectivity is obtained with the LiChrosorb diol but the efficiency remains very low.

As has already been demonstrated, the Deltabond CN phase is very deactivated and allows the elution of very polar compounds without adding modifiers [23–25]. For SubFC of amino acids, a minimum content of *ca.* 10% of modifier is required to dissolve samples in the mobile phase. With lower contents, the elution peaks exhibit strong front tailing. It appears that with such an amount of polar compounds in CO_2, retention of amino acids cannot be obtained on Deltabond CN. This means that

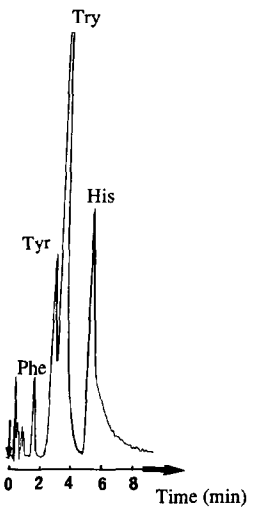

Fig. 1. SubFC of a mixture of four amino acids. Column, 150 × 4.6 mm I.D. LiChrosorb diol, 5 μm; mobile phase, CO_2–polar modifier (70:30, v/v); flow-rate, 2.8 ml/min (at 0°C); polar modifier, methanol–water–triethylamine (95:4.95:0.05, v/v/v); inlet pressure, 307 bar; outlet pressure, 250 bar; temperature, 30°C; UV detection at 225 nm, 0.08 a.u.f.s.

the amount of bonded CN groups is very low and/or the retention on other cyano-bonded phases used came from residual silanol groups.

Consequently, for further experiments, the basic modifier combined with LiChrosorb diol was chosen because partial resolution of mixtures of amino acids could be obtained and the efficiency was enhanced (Fig. 1). As described in the Introduction, subcritical conditions are only reached with 10–20% modifier.

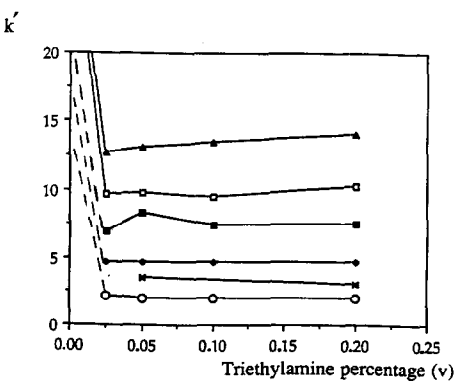

Fig. 2. Influence of triethylamine content in mobile phase on the retention of amino acids. Conditions as in Fig. 1, except CO_2–polar modifier (80:20, v/v); polar modifier, methanol–water–triethylamine (95 − x:5:x, v/v/v); UV detection at 254 nm; amount injected, 20 μl at 10^{-2} M. ○ = Leu; × = Val; ◇ = Pro; ■ = Ala; □ = Thr; ▲ = Hyp.

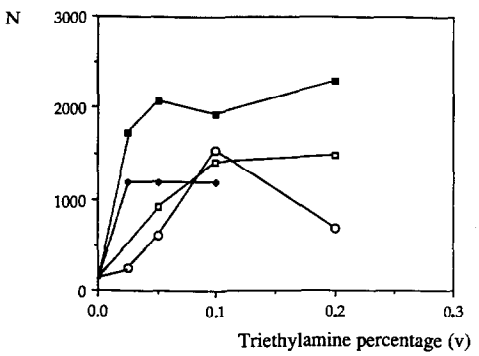

Fig. 3. Influence of triethylamine content in the mobile phase on the retention of amino acids. Conditions as in Fig. 2. ○ = Leu. ◇ = Pro; ■ = Ala; □ = Thr.

Improvement of separation efficiency on diol-bonded silica

Alcohol modifier. Methanol and 2-propanol were compared. Methanol was preferred although the chromatographic behaviour of the two alcohols were similar, the solubility of amino acids was higher in methanol.

Water modifier. The amount of water in the modifier between 1.5 and 11% (v/v) was investigated. No modification of solute retention with varying amount of water in the modifier could be observed. The percentage of water was fixed at 5%, mainly to

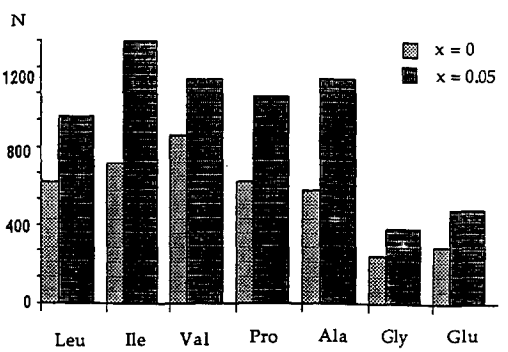

Fig. 4. Influence of triethylamine content in the mobile phase on column efficiency with pyridine modifier. Conditions as in Fig. 1 except CO_2–modifier (67:33, v/v); polar modifier, methanol–water–triethylamine–pyridine (82.8 − x:7.2:x:10, v/v/v); flow-rate, 4.5 ml/min (at 0°C); inlet pressure, 265 bar; detection, ELSD.

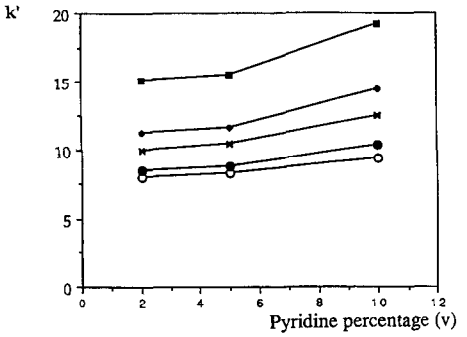

Fig. 5. Influence of pyridine content in the mobile phase on the retention of amino acids. Conditions as Fig. 1 except polar modifier, methanol–water–triethylamine–pyridine (93 − *x*:7:0.05:*x*, v/v); flow-rate, 5 ml/min (at 0°C); detection, ELSD. ○ = Leu; ● = Ile; × = Val; ◇ = Pro; ■ = Ala.

ensure solubility of amino acids in the mobile phase without demixing of the mobile phase.

Basic component. Experiments were carried out to study the influence of basic additives on retention, selectivity and efficiency; propylamine and pyridine were compared with triethylamine.

Propylamine exhibits almost the same behaviour as triethylamine (Fig. 2). Addition of a small amount (0.05%, v/v) of amine to the mobile phase greatly decreases the retention (by more than a factor of 2), this effect being more pronounced with triethylamine, whereas the selectivity does not vary. The influence of the amount of amine in the mobile phase on the efficiency is important, as shown in Fig. 3; the efficiency increases from a few hundred to 2000 TP by adding 0.05% (v/v) amine to the mobile phase.

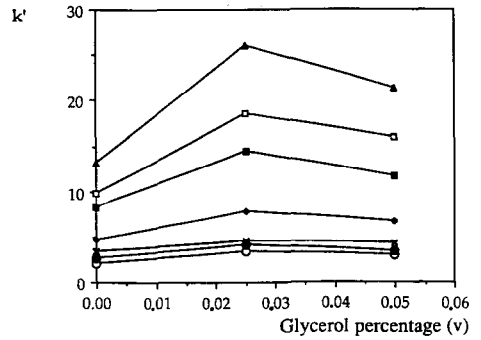

Fig. 6. Influence of glycerol content in the mobile phase on the retention of amino acids. Conditions as in Fig. 1, except polar modifier, methanol–water–triethylamine–glycerol (95.2 − *x*: 4.75:0.05:*x*, v/v); flow-rate, 4 ml/min; temperature, 40°C; outlet pressure, 200 bar; UV detection at 254 nm. ○ = Leu; ● = Ile; × = Val; ◇ = Pro; ■ = Ala; □ = Thr; ▲ = Hyp.

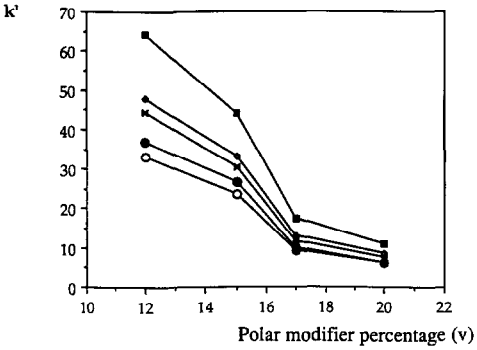

Fig. 7. Variation of capacity factors of amino acids with the amount of modifier in the mobile phase. Column, 50 × 4.6 mm I.D. LiChrosorb diol, 5 μm; mobile phase, CO_2–polar modifier (100 − *x*:*x*, v/v); flow-rate, 3 ml/min (at 0°C); polar modifier, methanol–water–triethylamine–pyridine (88:6.95:0.05:5, v/v); inlet pressure, 135 bar; temperature, 30°C; detection, ELSD. ○ = Leu; ● = Ile; × = Val; ◇ = Pro; ■ = Ala.

When triethylamine is replaced with pyridine, the percentage of the latter must be seven times higher to obtain a similar separation. The efficiency is improved by a factor of 2 if triethylamine is also added (Fig. 4); this indicates (i) the possibility of very strong interactions between the amino acids and the residual silanol groups and (ii) an easier separation of negatively charged amino acids, both requiring a more basic additive than pyridine. Fig. 5 indicates that the retention increases as the pyridine content in the mobile phase increases, in the presence of triethylamine. The latter observation is consistent

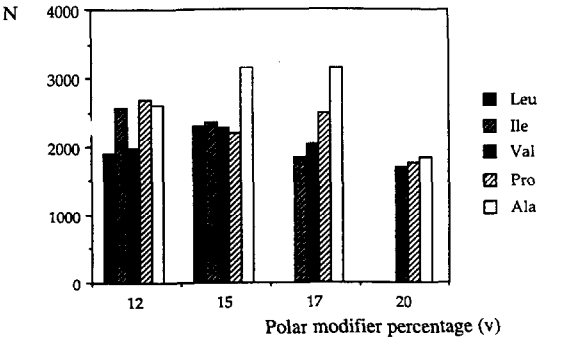

Fig. 8. Variation of efficiency with the amount of modifier in the mobile phase. Conditions as in Fig. 7.

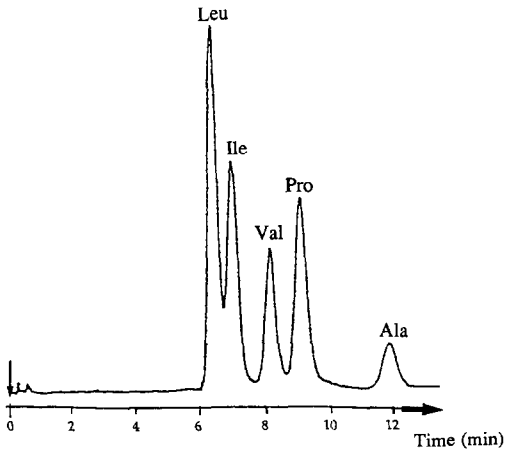

Fig. 9. SubFC of a standard mixture of five amino acids. Conditions as in Fig. 7, except CO_2–modifier (85:15, v/v); modifier, methanol–water–triethylamine–pyridine (87.95:7:0.05:5, v/v); flow-rate, 2.5 ml/min (at 0°C); inlet pressure, 107 bar.

with Janicot *et al.*'s results indicating impregnation of the stationary phase by the amine component of the modifer [15]. Here, both pyridine and triethylamine impregnate the diol-bonded silica. A similar effect is also obtained when ethylene glycol or glycerol [0–0.2% (v/v) in the mobile phase] is added to

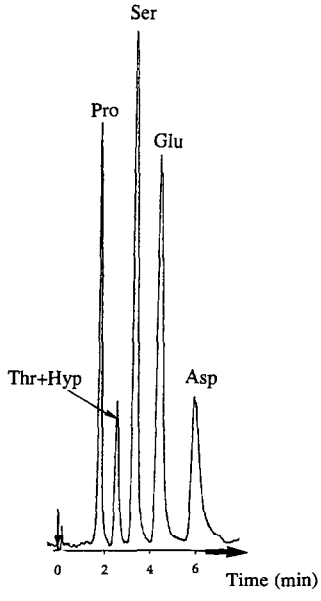

Fig. 10. SubFC of diacidic and hydroxy amino acids. Conditions as in Fig. 9, except CO_2–modifier (80:20, v/v); flow-rate, 5 ml/min (at 0°C); inlet pressure, 230 bar.

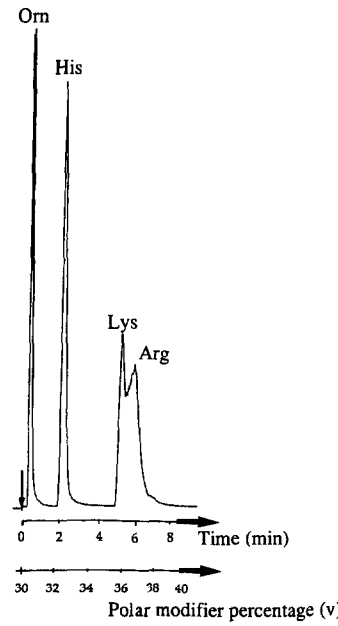

Fig. 11. SubFC of dibasic amino acids. Conditions as in Fig. 10, except modifier gradient from 30 to 40% (v/v) in CO_2.

the modifier instead of pyridine; the efficiency and selectivity are not modified and the efficiency ranges from 1000 to 2200 TP. Modification of the stationary phase is also evident from the curves showing an increase in retention with increasing amount of glycerol added (Fig. 6). Similar results were reported recently by Smith [26].

In conclusion, the following quaternary mixture was chosen as the "modifier": methanol–water–triethylamine–pyridine (87.95:7:0.05:5, v/v). It provides good efficiency [3.5 (at 2.5 ml/min) $< h <$ 10, h = reduced plate height] and acceptable retention of amino acids.

Examples of separations

By varying the content of the modifier in the CO_2, the retention (Fig. 7) and efficiency (Fig. 8) can be adjusted to maximize the column efficiency and to obtain a good separation.

Thus, using a short column and a CO_2–modifier mixture (85:15, v/v), less polar amino acids can be easily resolved (Fig. 9). The elution order and the polarity (Rekker indices) are well correlated.

Increasing the modifier content to 20% permitted the separation of more polar amino acids including

diacids (glutamic and aspartic acid, Fig. 10). Under these conditions threonine and hydroxyproline co-elute without any possibility of resolving them by varying the proportions of the modifier or the CO_2 density.

Elution of dibasic amino acids can even be obtained by packed column SubFC but it requires a gradient elution from 30% to 40%. With this high modifier content, arginine is still very difficult to elute and the peak exhibits strong front tailing (Fig. 11).

Owing to the short length of the column used and the high linear velocity of the mobile phase, the analysis times remain low in spite of the retention of the amino acids (the k' values always exceed 10). Fast re-equilibration of the column was observed in accordance with Steuer *et al.*'s data [27] (10–30 column volumes, *ca.* 5 min). No regeneration test of the columns was undertaken.

CONCLUSIONS

SubFC–ELSD allows separations of amino acids without any derivatization step on diol-bonded silica. Various stationary and mobile phases were investigated to enhance the efficiency, which was the major problem with which we had to deal. It requires impregnation of the stationary phase with basic additives or polyols. Samples containing five or six amino acids have been partially resolved. Using a modifier gradient, resolution of more than ten amino acids could certainly be obtained in less than 10 min.

These results cannot compete with LC coupled with modern derivatization techniques, but they highlight the wide potential of SubFC on packed columns for the separation of medium polarity molecules. These can be separated more easily than amino acids by LC or SubFC using conventional modifiers, the latter being much faster and cheaper owing to the time saved and the lower price of industrial CO_2 in comparison with LC solvents. Hence there is no major reason why routine analyses of medium polarity solutes could not be done by SubFC rather than LC.

ACKNOWLEDGEMENTS

The authors thank Rexim for initiating and financially supporting this work and Gilson France for the loan of the prototype supercritical fluid chromatograph.

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