Journal of Chromatography, 384 (1987) 249–258
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1033

FACTORS CONTROLLING THE SEPARATION OF AMINO ACIDS IN ISOCRATIC REVERSED-PHASE LIQUID CHROMATOGRAPHY

SHULAMIT LEVIN and ELI GRUSHKA*
Department of Inorganic and Analytical Chemistry, The Hebrew University, Jerusalem (Israel)

SUMMARY

Amino acids can be separated with a simple isocratic liquid chromatographic system in which no pre- or post-column derivatization of the amino acids is needed. Copper ions and alkylsulphonate additives are used to effect both the selectivity and the retention of the solutes. The effect of several operating conditions on the performance of the system was examined, especially the nature and concentration of the alkylsulphonate, the concentration of copper ions, the ionic strength of the buffer and temperature. Guidelines are given for optimization of the separation. The deleterious effects of system peaks and means of overcoming them are discussed.

INTRODUCTION

We recently described a method for the separation of amino acids[1] in which the mobile phase was an aqueous acetate buffer containing alkylsulphonate and copper(II) acetate. A reversed-phase column and UV detection were used. The two major features of the method are that elution is isocratic and that it is unnecessary to derivatize the amino acids. The chromatographic equipment is readily assembled, highly reproducible and inexpensive.

The rationale behind the choice of the mobile phase constituents has been described in detail[1]. However, for the sake of completeness, it should be mentioned here that copper ions were added to control the detection and retention of the amino acids, and the alkylsulphonate was added to retard the early eluting solutes. Some of the technical problems related to this particular chromatographic system and the influence of the chromatographic parameters on the retentions and selectivities of several representative amino acids were also discussed[1].

This work intends to illustrate the usefulness of this chromatographic system by examining the influence of some chromatographic parameters on the chromatogram, rather than on the behaviour of individual solutes, as was done previously[1]. The effects of varying parameters such as the alkylsulphonate chain length and concentration, Cu^{2+} concentration, ionic strength and temperature were studied. These parameters affect the retention and resolution considerably.

0021-9673/87/$03.50

EXPERIMENTAL

As the experimental setup was described in detail previously[1], only a brief description is given here.

Materials

The mobile phase was prepared by dissolving the appropriate amounts of a given alkylsulphonate and copper(II) acetate in an acetate buffer (pH 5.6). All the alkylsulphonates and amino acids were purchased from Sigma (St. Louis, MO, U.S.A.).

Instrumentation

The chromatographic system consisted of a Perkin-Elmer (Norwalk, CT, U.S.A.) Series 4 liquid chromatograph, a Rheodyne (Cotati, CA, U.S.A.) injection valve and a Perkin-Elmer 85B variable-wavelength spectrophotometric detector. The column temperature was maintained with the aid of a thermostated water-bath. All the chromatographic analyses were carried out using a Merck (Darmstadt, F.R.G.) LiChrosorb RP-18 cartridge (250 × 4 mm I.D.).

Procedures

The flow-rate of the mobile phase was 2 ml/min. Detection was carried out at 235 nm. The concentrations of the injected amino acids were 2.4–10 nmole, depending on the elution time of the solutes.

RESULTS AND DISCUSSION

Effect of chain length of the alkylsulphonate

It was shown previously that the retention of the amino acids, at a given sulphonate concentration, increases as the length of the alkyl portion of the sulphonate is increased[1]. This is to be expected, as the retention processes here are identical with those in a more conventional ion-pair system[2]. Hence the same number of amino acids can be separated in roughly the same time by using different concentrations of

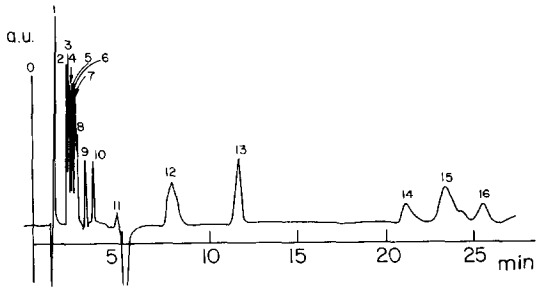

Fig. 1. Separation of amino acids with pentanesulphonate. Mobile phase, 10 m*M* acetate buffer–0.5 m*M* copper(II) acetate–5 m*M* pentanesulphonate. Detection, 235 nm, 0.32 a.u.f.s.; amino acid concentrations, 0.5–1 m*M*; flow-rate, 2 ml/min; temperature, 30°C. Peaks: 1 = Asp + Glu; 2 = Gly; 3 = Ser; 4 = Asn; 5 = Ala; 6 = Gln; 7 = Hyp; 8 = Thr; 9 = His; 10 = αAbu; 11 = Pro; 12 = Val + Nvl + Lys; 13 = Met; 14 = Arg; 15 = Tyr + Ile; 16 = Leu.

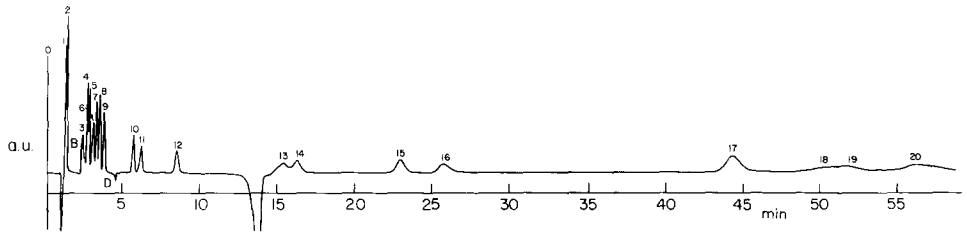

Fig. 2. Separation of amino acids with hexanesulphonate. Mobile phase, 10 m*M* acetate buffer–0.5 m*M* copper(II) acetate–5 m*M* hexanesulphonate. Other conditions as in Fig. 1. Peaks: 1 = Asp; 2 = Glu; 3 = Gly; 4 = Ser; 5 = Asn; 6 = Ala; 7 = Gln; 8 = Thr; 9 = Hyp; 10 = αAbu; 11 = His; 12 = Pro; 13 = Val; 14 = Nvl; 15 = Met; 16 = Lys; 17 = Tyr; 18 = Ile; 19 = Leu; 20 = Arg.

the various alkylsulphonates. For example, it was found that mobile phases containing either 0.8 m*M* heptanesulphonate, 4 m*M* hexanesulphonate or 10 m*M* pentanesulphonate in the mobile phase gave similar chromatograms. This finding is important in the optimization of the method.

In the previous study we investigated the use of C_6–C_8 sulphonates; this work extends the method to pentanesulphonate. Fig. 1 shows a chromatogram with 16 peaks, all eluted in less than 30 min. However, the early part of the chromatogram is crowded, 11 peaks being eluted in less than 5 min. Moreover, some of the peaks are actually due to mixtures of several amino acids. The use of hexanesulphonate can alleviate these difficulties, as shown in Fig. 2. The chromatographic conditions for Figs. 1 and 2 were identical, except for the chain length of the ion-pairing reagent. The resolution improved considerably with the introduction of the longer chain sulphonate, but the retention time also increased. For example, with pentanesulphonate in the mobile phase, leucine, which is the last solute in Fig. 1, was eluted in about 25 min whereas with hexanesulphonate in the mobile phase it was retained for over 50 min.

Effect of sulphonate concentration

The behaviour of some representative amino acids as a function of the sulphonate concentration was examined previously[1]. Studies in which hexanesulpho-

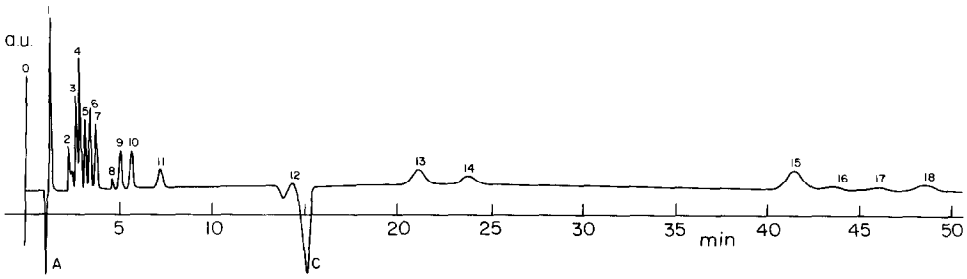

Fig. 3. Effect of Cu^{2+} and sulphonate concentration on the separation of amino acids. Mobile phase, 10 m*M* acetate buffer–0.25 m*M* copper(II) acetate–5 m*M* hexanesulphonate. Other conditions as in Fig. 1. Peaks: 1 = Asp + Glu; 2 = Gly; 3 = Ser; 4 = Asn + Ala; 5 = Gln; 6 = Thr; 7 = Hyp; 8 = system peak D; 9 = αAbu; 10 = His; 11 = Pro; 12 = Val + Nvl; 13 = Met; 14 = Lys; 15 = Tyr; 16 = Ile; 17 = Leu; 18 = Arg.

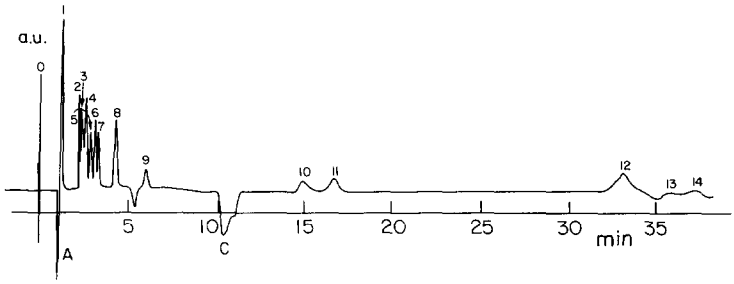

Fig. 4. Effect of sulphonate concentration on the separation of amino acids. Mobile phase, 10 m*M* acetate buffer–0.25 m*M* copper(II) acetate–2.5 m*M* hexanesulphonate. Other conditions as in Fig. 1. Peaks: 1 = Asp + Glu; 2 = Gly + Ser; 3 = Asn; 4 = Ala; 5 = Gln; 6 = Thr; 7 = Hyp; 8 = αAbu + His; 9 = Pro; 10 = Lys; 11 = Met; 12 = Tyr + Arg; 13 = Ile; 14 = Leu.

nate, heptanesulphonate and octanesulphonate were used as mobile phase additives showed similar patterns of solute behaviour. As the sulphonate concentration increased, so did the retention of the test solutes. This is further demonstrated in Figs. 3 and 4. In these two instances, the same concentration (and pH) of acetate buffer and of copper ions were used, *viz.*, 10 m*M* and 0.25 m*M*, respectively. However, in the first instance the sulphonate concentration was 5 m*M*, whereas in the second it was 2.5 m*M*. The general increase in retention times and the improved resolutions are evident from Figs. 3. For example, the peaks due to αAbu and His are well resolved as peaks 9 and 10 in Fig. 3, but when the concentration of the sulphonate is halved (Fig. 4) they are eluted in single peak (peak 8).

Several other interesting features can be observed in Figs. 3 and 4. First, the Val and Nvl peaks are masked by a system peak (peak C) in both chromatograms. In Fig. 3 they are indicated as peak 12, whereas in Fig. 4, where they are completely overlapped by system peak C, they are not indicated. The increase in the retention times of the two amino acids and of the system peak, C, are fortuitously identical as the concentration of the sulphonate is increased from 2.5 to 5 m*M*. Second, the behaviour of the polar amino acids is strongly influenced by the concentration of the sulphonate. The resolution among the members of this group is better when higher concentrations of sulphonate are used. Finally, Arg is very sensitive to the sulphonate concentration[1]. It appears as the last-eluted solute when 5 m*M* sulphonate is used (peak 18 in Fig. 3, following the Leu and the Ile peaks). However, at a lower sulphonate concentration its retention is decreased, so that it precedes the Leu and Ile peaks, and merges with Tyr to yield peak 12 in Fig. 4.

All of the above observations can be explained in terms of the retention processes that occur in the column. It is assumed that the copper ions form a charged complex with the amino acids, which in turn forms an ion-pair complex with the alkylsulphonate. Hence the polarity of the amino acids determines the extent of interaction with the sulphonate, and therefore the retention in the column. Moreover, the basic amino acids, being positively charged under the conditions of the experiment, should interact very strongly with the negatively charged sulphonate. This was verified experimentally.

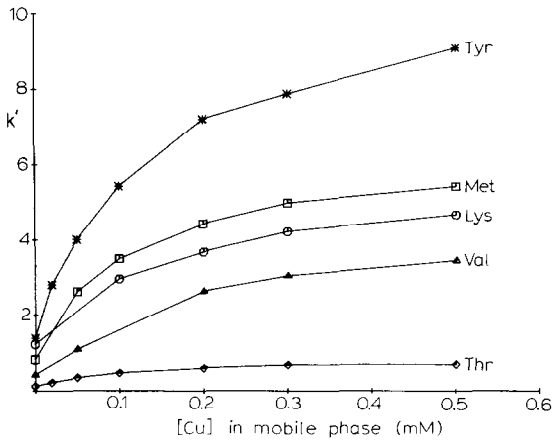

Fig. 5. Dependence of retention on Cu^{2+} concentration. Mobile phase, 0.1 *M* acetate buffer–5 m*M* heptanesulphonate with various concentrations of copper(II) acetate. Temperature, 30°C; flow-rate, 2 ml/min; concentration of amino acids, 0.4 m*M*.

Effect of copper concentration

The copper ions, having the unique ability of complexing amino acids, are essential to the method and the copper concentration in the mobile phase is a very important parameter. The retention of five representative amino acids as a function of the copper content in the mobile phase is illustrated in Fig. 5. The concentration of all the amino acids was 0.4 m*M* and that of copper in the mobile phase was varied from 0 to 0.5 m*M*. The acetate buffer and the concentration of the sulphonate were kept constant throughout this set of experiments.

In this range of concentrations, the resulting amino acid–copper complex is an equimolar complex. Hence in the experiments depicted in Fig. 5, when the copper concentration was increased the extent of formation of the copper–amino acids complex also increased. This, in turn, resulted in longer retention times up to a certain copper concentration, above which no further increase in the complex concentration and therefore no retention changes will be observed. This explains the saturation shape of the curves displayed in Fig. 5. These curves can be utilized to calculate the formation constants of the amino acid–Cu^{2+}–alkylsulphonate ternary complex using the methodology developed by Horváth *et al.*[3] and Grushka and Cohen[4]. The situation in the present instance is complicated by the fact that the sulphonate extracts copper ions into the stationary phase, making the mathematics more complicated. This point will be dealt with in a subsequent publication.

The effect of the copper concentration in the mobile phase on the whole chromatogram is shown in Figs. 2 and 3. Two cases are presented in which the buffer and the sulphonate concentrations were kept constant while the copper concentration was changed. In one instance 0.5 m*M* copper was used (Fig. 2) and in the other 0.25 m*M* copper was used (Fig. 3). An improvement in the resolution is obtained at the higher copper concentration, but the analysis time is increased. The improvement in resolution is most notable with the polar amino acids, as was observed in the study of the sulphonate concentration. An example is Asp and Glu. Whereas these two amino acids can be resolved at the higher concentration of copper (peaks 1 and 2 in

Fig. 2), no resolution is observed at the lower concentration (Fig. 3). Asn and Ala exhibit the same behaviour: they are separated at the higher copper concentration (peaks 4 and 5 in Fig. 2), whereas at the lower copper concentration they are unresolved (peak 4 in Fig. 3). Val and Nvl are separated at the higher copper concentration and appear in the chromatogram following the system peak (indicated by C). However, at the lower copper concentration, Val and Nvl are eluted earlier, while the retention time of the system peak increases a slightly little. The result is overlapping of these peaks, and the solutes are masked by the intense system peak.

The copper ions have an additional role in addition to controlling the retention, *i.e.*, they aid in the detection by the virtue of the absorbance of the charge-transfer complex with the amino acid. In general, as the copper ion concentration is increased the detection limits improve. However, this improvement is counterbalanced by the fact that the increasing copper concentration also increases the retention. As the elution is isocratic, the peaks of the strongly retained solutes are very broad, and their detection limits are far poorer than those of the fast-eluted solutes. Typical detection limits for the less retained amino acids are around 10^{-5} *M*, whereas they are an order of magnitude higher for the strongly retained amino acids.

Effect of ionic strength

The retention of the amino acids in the present system also depends on the ionic strength. The behaviour of several representative amino acids as a function of ionic strength has been investigated previously[1]. It was shown that the retention times decreased sharply as the concentration of the acetate buffer increased up to 0.1 *M*. At higher buffer concentrations the changes in the retention times were much more moderate.

Figs. 4 and 6 show examples of the influence of the concentration of the acetate buffer on the chromatograms as a whole. Two cases are presented. The sulphonate and the copper concentrations were kept constant at 2.5 and 0.25 m*M*, respectively, in both instances. The chromatogram shown in Fig. 4 was obtained with a 10 m*M* acetate buffer, whereas for Fig. 6 a 25 m*M* acetate buffer was used. The first notable

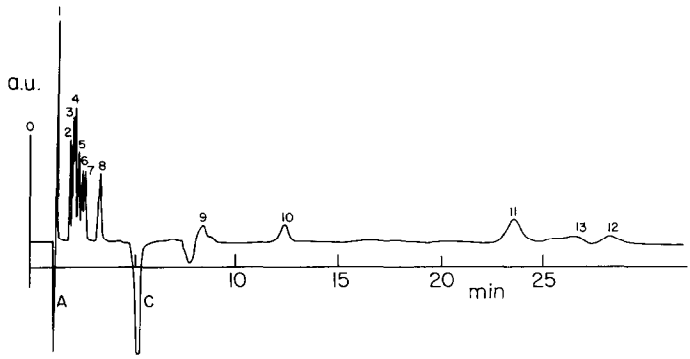

Fig. 6. Effect of ionic strength on the separation of amino acids. Mobile phase, 25 m*M* acetate buffer–0.25 m*M* copper(II) acetate–2.5 mM hexanesulphonate. Other conditions as in Fig. 1. Peaks: 1 = Asp + Glu; 2 = Gly; 3 = Ser; 4 = Gln + Asn; 5 = Ala; 6 = Thr; 7 = Hyp; 8 = αAbu + His; 9 = Lys + Val + Nvl; 10 = Met; 11 = Tyr; 12 = Leu; 13 Ile + Arg.

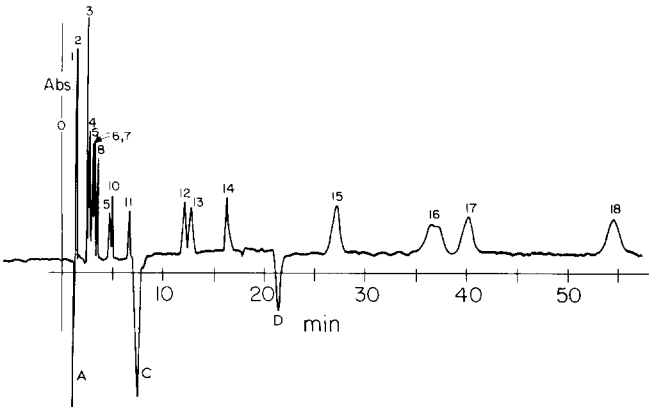

Fig. 7. Chromatogram obtained at 40°C. Mobile phase, 10 m*M* acetate buffer–0.4 m*M* copper(II) acetate–0.8 m*M* heptanesulfonate. Other conditions as in Fig. 1. Peaks: 1 = Asp; 2 = Glu; 3 = Gly + Ser; 4 = Asn; 5 = Gln; 6 = Thr; 7 = Ala; 8 = Thr; 9 = αAbu; 10 = His; 11 = Pro; 12 = Val; 13 = Nvl; 14 = Met; 15 = Tyr; 16 = Ile; 17 = Leu; 18 = Arg.

feature is the interference of the system peaks, which is more serious at the higher buffer concentration. As some of the system peaks appear in the middle range of the chromatogram, they may mask some amino acids such as Pro, Lys, Val and Nvl. For example, the Pro and Lys peaks (peaks 9 and 10, respectively, in Fig. 4) are absent from Fig. 6, being completely masked by the adjacent system peaks. As mentioned before, the Val and Nvl peaks are masked in both instances.

The general trend of the data shows a non-selective decrease in retention with increasing ionic strength. The resolution is relatively independent of the ionic strength, especially at buffer concentrations higher than 0.1 *M*. The different number of separable solutes is a result of the interference of the system peaks rather than changes in the retention times of the solutes.

Effect of temperature

The influence of temperature on the retention of the amino acids in the present system was briefly examined previously[1]. The general trend observed was a selective

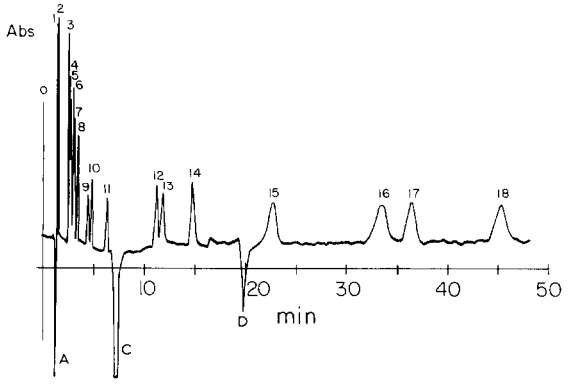

Fig. 8. Chromatogram obtained at 45°C. Conditions and peaks as in Fig. 7.

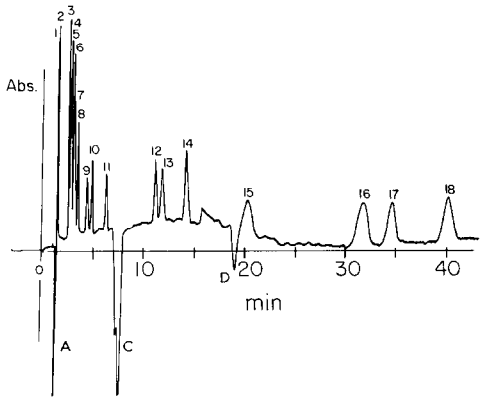

Fig. 9. Chromatogram obtained at 48°C. Conditions and peaks as in Fig. 7.

decrease in the retention times of the amino acids when the temperature was increased. The effect of temperature changes on the whole chromatogram is shown in Figs. 7–9. An identical mixture of amino acids was chromatographed in three systems, each containing 10 m*M* acetate buffer, 0.8 m*M* heptanesulphonate and 0.4 m*M* copper(II) acetate, but at different temperatures. Although the temperature range studied covered only 8°C, the system is sensitive enough to demonstrate a relatively large effect. The general trend is as expected, a decrease in retention times as the temperature is increased. The analysis time for the separation of 18 peaks decreased from 60 min at 40°C to about 40 min at 48°C. However, this is a small enough temperature range so that the selectivities, and hence the resolutions, do not change appreciably. Therefore, temperature can be an important parameter in the optimization of the separation of amino acids. An important effect of temperature on the chromatographic baseline is exhibited in these figures. At higher temperatures the baseline instability is marked, which results in poorer detection limits, partly offsetting the gain in the analysis times.

The problem of the system peaks

The solutes were injected into the column as solutions in pure water. This gave rise to four system peaks in the present system, in addition to peaks due to the solutes. The system peaks are present in all chromatograms, but are best seen in Figs. 7–9, where three of them (A, C and D) are clearly observed. The origins and the processes leading to the formation of these system peaks are discussed elsewhere[5]. They were found to be due to sodium, copper(II) and sulphonate ions, respectively. The fourth system peak (which is present but not visible in the chromatograms shown here) is probably related to the acetate. Using the terminology of ref. 5, it will be called system peak B. Peaks A, C and D are usually negative, whereas peak B is positive in the concentration range of solutes and mobile phase additives employed in this separation system.

Although the information conveyed by the system peaks is extremely useful, they can interfere with the chemical analysis. Of the four system peaks in the present study, peak A is eluted before all the solutes. In fact, it can be employed as the void volume marker. Peak B is usually small and is eluted with the polar amino acids

group. These two system peaks are fairly insensitive to changes in the experimental conditions and therefore do not interfere seriously with the identification or the determination of the amino acids. On the other hand, peak C, which is attributed to the copper ions, is very sensitive to changes in the chromatographic parameters and to the sample concentration. It may appear as an intensely negative peak and, as it may overlap with solute peaks, the latter can be completely masked. System peak D is related to the presence of the sulphonate ions. Although when hexanesulphonate is used peak D is not much influenced by the solute concentration, it may be sufficiently negative to mask adjacent solutes effectively.

The solution to this interference problem lies in an understanding of the nature of these peaks and the reasons for their specific behaviour[5]. When the interference is due to the copper peak (peak C), the sample should contain sufficient copper ions, roughly similr to the total concentration of the injected amino acids. This will compensate for the vacancy of the copper(II) in the sample zone. When peak D causes the difficulties, the solution is even simpler. The sulphonate should be present in the injected sample at roughly the same concentration as in the mobile phase. The old adage of liquid chromatography that the sample should be dissolved in, and diluted with, the mobile phase is particularly true for the system described here. In fact, it was found that frequently the sample had to be enriched with respect to some of the mobile phase components. This was essential in order to counteract the possibly severe local equilibrium disturbances due to the strong interactions between the solutes and the mobile phase components.

CONCLUSION

Not all the 20 or so natural amino acids can be separated in one chromatographic run with the system described here. However, frequently a smaller subset of this group of amino acids needs to be separated. As the "fine tuning" of the selectivity of this system is easy and direct, it is simple to choose suitable conditions for any particular group of amino acids. When a mixture of hydrophobic and basic amino acids needs to be separated, the ionic strength of the mobile phase and the column temperature should be raised, whereas the concentrations of copper and sulphonate should be lowered. On the other hand, when the mixture contains mostly polar, acidic or small amino acids, the above changes should be reversed.

There are several other attractive features of the method. As elution is isocratic and pre- or post-column derivatization of the amino acids is not required, the chromatographic setup is simple and is readily constructed. It includes the minimum number of components essential for an HPLC system: one reservoir, a simple pump, a conventional RP-18 column (40 000 plates/m is sufficient) and a UV detector capable of operating at 235 nm. The equilibration time of the column is short. The linearity of the quantitative determination is excellent, provided that the sample is injected at the appropriate concentrations range[1]. However, several precautions must be taken in order to overcome some practical problems related to the use of copper(II) ions in the mobile phase. These are detailed in our previous paper[1].

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