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AMPEROMETRIC DETECTION OF AMINO ACIDS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A COPPER ELECTRODE

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SUMMARY

A copper electrode has been used as an amperometric detector for amino acids in high-performance liquid chromatography. The amino acids are separated in a reversed-phase system, using silica-based and polymer-type column materials. Neutral or alkaline buffer solutions of phosphate and carbonate can be used as mobile phases. Borate buffers are less suitable.

The detection method is characterized by a high linear dynamic range, good reproducibility, the absence of electrode poisoning and a sensitivity comparable to that of UV absorption methods after derivatization of the amino acids. Detection limits with conventional-scale columns are in the range 10–100 pmoles.

A reduction in the flow-rate in the flow-through cell improves the sensitivity for amino acids that give relatively low signals, such as proline. Therefore, the use of microbore columns is especially advantageous for these compounds. The absolute detection limits decrease by about one order of magnitude on changing to a miniaturized system.

INTRODUCTION

The separation of amino acids by liquid chromatography has been accomplished in many ways. Separation by means of "classical" ion-exchange chromatography is a well established method. Spectrophotometric detection with ninhydrin is widely used (*e.g.*, ref. 1), but other methods have also been developed, such as fluorimetric detection with an *o*-phthalaldehyde (OPA) reagent[2–4].

Another widely used approach is pre-column derivatization of the amino acids, whereupon the derivatives are separated by reversed-phase high-performance liquid chromatography (HPLC). Commonly used are derivatives with phenylthiohydantoin (PTH) (*e.g.*, refs. 5–10), 1,4-dinitrophenol (DNP)[11], Dns chloride[12–14] and OPA[15–17]. These derivatives can be detected by UV absorbance (PTH, DNP) or fluorimetric (Dns, OPA) measurements.

Recently, the separation of underivatized amino acids has again attracted attention. However, reversed-phase HPLC columns instead of the less efficient ion-

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exchange columns are now more frequently used. This is a consequence of the development of ion-pair chromatography as a separation technique[18,19] and progress in post-column reaction detection, where the amino acids are derivatized after the separation. Although sensitive detection of underivatized amino acids can be achieved by UV absorption at a wavelength of about 200 nm[20], the selectivity is much improved by this post-column derivatization with, *e.g.*, ninhydrin or OPA.

Another selective detection method for underivatized amino acids is potentiometry with a copper-selective[21] or copper electrode[22,23]. The main drawback of this method is that the detector response is not linear with the amino acid concentration. Also, the sensitivity for different amino acids varies, which has not been explained satisfactorily.

In an earlier part of this study[24], using a rotating disk electrode, it was shown that the current flowing through a copper anode in a neutral or alkaline buffer solution increased linearly with increasing concentration of amino acids in the solution. It was shown that this was due to increased dissolution of a copper(II) oxide or hydroxide layer formed on the surface of the electrode, by the complex formation of Cu(II) ions with amino acids. The difference in sensitivity for different amino acids was caused by a difference in complexation rate. A theoretical model describing the dependence of the current on the reaction rate was verified experimentally.

In the work presented in this paper, we applied the results obtained with a rotating disk electrode to a flow-through cell with a copper electrode as an amperometric detector in HPLC. The detector performance was studied under various chromatographic conditions and compared with that of other detection methods.

EXPERIMENTAL

Apparatus

Mobile phase flow-rates of between 0.1 and 2.0 ml/min were delivered by a Waters Assoc. (Milford, MA, U.S.A.) 6000 A pump. Samples were injected with a Rheodyne (Berkeley, CA, U.S.A.) valve, with 7-, 20-, 25- and 1000-μl loops. The copper electrode, with a diameter of 3 mm, was housed in a Metrohm (Herisau, Switzerland) EA 1096/2 flow-through cell, with a platinum auxiliary electrode and silver–silver chloride–1 *M* lithium chloride in methanol–water (1:1) as reference electrode. The potential of the working electrode was kept at +100 mV. The copper electrode was polished regularly with Gamal (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and rinsed with 5 *N* nitric acid. Before a series of experiments the electrode potential was set at −300 mV for 5 min and at +100 mV for at least 15 min. Currents were measured with a home-made potentiostat–amplifier and recorded with a Kipp & Zonen (Delft, The Netherlands) BD 10 recorder.

Reagents

L-Amino acids (Sigma grade) were obtained from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical-reagent grade. Mobile phases were degassed before and during experiments by purging with nitrogen at 30°C.

Chromatographic columns

A 250 × 4.6 mm I.D. column packed with LiChrosorb RP-18 (5 μm) (Merck,

Darmstadt, G.F.R.) was obtained from Supelco (Bellefonte, PA, U.S.A.). Microbore columns (250 × 1.1 mm I.D.) were constructed and packed with 7.7 μm LiChrosorb RP-18 as described previously[25]. For the packing of a microbore PRP-1 column, a packing solution of 10% glycerol and 3% potassium nitrate in water was prepared. A 0.25-g amount of PRP-1 (Hamilton, Reno, NE, U.S.A.) was wetted with a few drops of acetone and a slurry was made with 2 ml of the packing solution by stirring in an ultrasonic bath. The microbore column was packed by pumping the packing solution for 20 min at a constant pressure of 350 bar.

RESULTS AND DISCUSSION

Separation with phosphate buffers

In our investigations with the rotating disk electrode[24], we have found that the ratio of the current increase due to the presence of amino acid to the background current in phosphate buffers was high at low buffer concentration and high pH. As the sensitivity of an HPLC detector is determined by the ratio of the signal (the current increase) to the noise, which is about proportional to the background current, these conditions will give optimum detector performance.

Fig. 1 shows the separation of the eight essential amino acids on a bonded-phase C-18 column, with a phosphate buffer of rather low concentration as the mobile phase. Six amino acids were well separated, but the almost non-retained compounds lysine and threonine were not. Decreasing the percentage of methanol in the mobile phase to 2.5% did not improve the separation of these two compounds.

The linear dynamic range of the detector was measured for 25-μl leucine samples. As can be seen in Fig. 2, the peak current (i_p) varied linearly with the sample concentration (c) from $3 \cdot 10^{-6}$ up to 10^{-3} M. In this range the peak heights measured

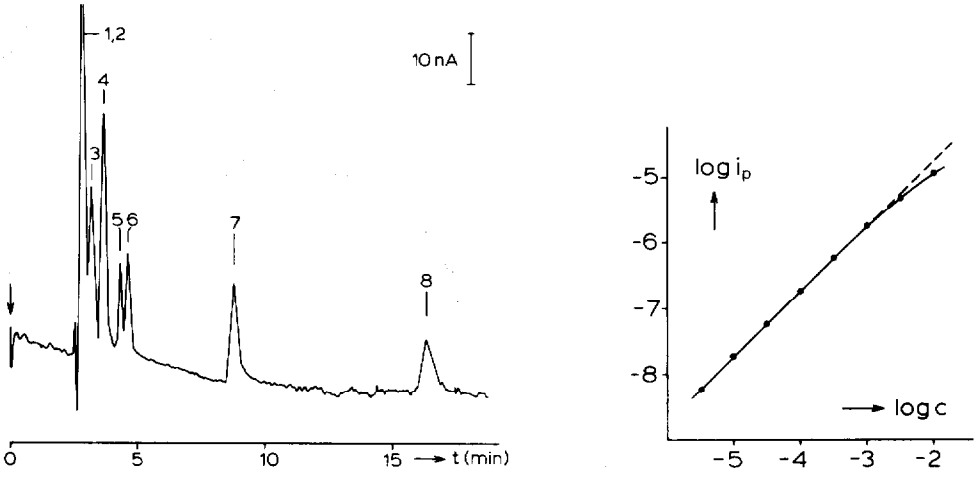

Fig. 1. Separation of the essential amino acids with phosphate buffer. Column: 250 × 4.6 mm I.D., 5 μm LiChrosorb RP-18. Mobile phase: 0.025 M phosphate buffer, 10% methanol, pH = 7.2. Flow-rate: 1.0 ml min^{-1}. Sample: 25 μl, 10^{-5} M of: (1) lysine; (2) threonine; (3) valine; (4) methionine; (5) isoleucine; (6) leucine; (7) phenylalanine; (8) tryptophan.

Fig. 2. Linear dynamic range of leucine with phosphate buffer. Conditions as in Fig. 1.

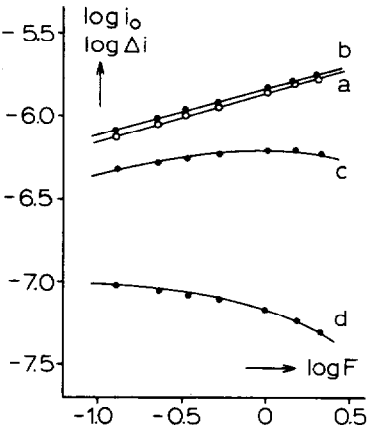

Fig. 3. Dependence of current on flow-rate: 1-ml plug injections with 0.025 *M* phosphate buffer, 10% methanol, pH 7.2. (a) Baseline current. Peak heights of 10^{-4} *M* histidine (b), alanine (c) and proline (d).

were 71 ± 2 A mole^{-1}. With a peak-to-peak noise of 1.0 nA, the lower limit of detection (signal-to-noise ratio = 2) was calculated to be 28 pmoles (4 ng).

The day-to-day variance of the peak height was about 7% (n = 10). This variance appeared to be due mainly to changes in the surface properties or the positioning of the electrode, as the deviations in one uninterrupted series of experiments were much smaller. The peak-height variance could be reduced to less then 3% if one of the amino acids in the mixture was used as an internal standard.

One of the major problems in electrochemical detection with solid electrodes is the poisoning of the electrode. With the copper electrode, however, this effect was not observed. Even repeated injections of notorious "poisoners", such as urine samples or cysteine solutions, did not reduce the sensitivity of the electrode. The good performance of the detection system in this respect can be explained by the fact that the outer surface of the electrode consists of a copper oxide or hydroxide layer which is slowly dissolving and therefore constantly renewed.

Use of microbore columns

In our work with the rotating disk electrode, the relationship between the currents and the rate of convection towards the electrode, governed by the rotation speed of the electrode, was investigated theoretically and experimentally. It was shown that the background current and the signal that is caused by a fast ($k_2 > 10^6$ mole^{-1} l sec^{-1}) complexation reaction of copper ions with an amino acid increase with increasing rotation speed. If the complexation reaction is slow ($k_2 < 10^4$–10^5 mole^{-1} l sec^{-1}), the current increase decreases with increasing rotation speed.

In a flow-through cell the parameter that is comparable to the rotation speed of a rotating disk electrode is the flow-rate. To investigate the dependence of the currents on the flow-rate we injected 1-ml plugs of mobile phase solutions containing amino acid and the maxima of the resulting current peaks at different flow-rates, F, were measured (Fig. 3). The graph of log i_0 (where i_0 is the baseline current) *versus* log F is a straight line with a slope 0.30. This value differs considerably from the values predicted theoretically for a wall-jet (0.75) or a thin-layer cell (0.50). It is also smaller

than the value of 0.44 measured before for the same cell geometry[26]. However, the last value was measured with an electrode of larger diameter (6 mm).

The peak height for the rapidly complexing amino acid histidine shows virtually the same dependence on the flow-rate as does the baseline current. For the slowly reacting proline the peak height decreases with increasing flow-rate. Obviously the signal-to-noise ratio for rapidly reacting compounds will not depend on the flow-rate, while for a slowly reacting amino acid the sensitivity can be improved by decreasing the flow-rate. The use of microbore columns will therefore be favourable especially for the detection of those compounds which give relatively low responses.

We have compared the sensitivities for a number of amino acids in a microbore system (column I.D. 1.1 mm) with those in a conventional system (column I.D. 4.6 mm). The comparison is influenced by several factors. (a) As mentioned above, the current increase due to a certain amino acid concentration is a function of the flow-rate. (b) The noise is also influenced by the flow-rate. At the flow-rate of 1.00 ml min^{-1} used with conventional columns, the baseline current was 1.35 μA and the peak-to-peak noise 1.0 nA. With the microbore columns a flow-rate of 0.13 ml min^{-1} was used and the baseline current was then 0.70 μA and the noise 0.6 nA. (c) Peak dilution by extra-column effects is significant when microbore columns are used, especially for early eluting compounds. The extra-column band broadening was measured as described earlier[25]; it was observed that with a sample volume of 7 μl, σ = 7.9 μl. (d) The commercial 4.6 mm I.D. column had a higher efficiency (number of plates, N = 7500) than the home-packed microbore column (N = 2600).

The first two factors favour the microbore system whereas the latter two have a negative effect. However, recently the packing technique for microbore columns was optimized[27]; now, microbore columns can be packed with an efficiency comparable to that of commercial 4.6 mm I.D. columns.

Apart from this, as usual peak dilution will be smaller in microbore columns owing to the smaller cross-sectional area of the column, and the absolute detection limit will be decreased proportionally. On the other hand, the sample volume in a miniaturized system must be reduced to avoid extensive extra-column band broaden-

TABLE I

COMPARISON OF SENSITIVITY WITH CONVENTIONAL AND MICROBORE COLUMNS

For conditions see text. k' = Capacity factor.

Amino acid	k'	*Response* ($A\ mol^{-1}\ l \cdot 10^{-3}$)		*Detection limit**			
				$mol\ l^{-1} \cdot 10^{-6}$		*ng*	
		4.6 mm I.D. column	*1.1 mm I.D. column*	*4.6 mm I.D. column*	*1.1 mm I.D. column*	*4.6 mm I.D. column*	*1.1 mm I.D. column*
Proline	0.14	0.56	1.08	3.6	1.1	10.3	0.9
Valine	0.27	2.40	2.28	0.8	0.5	2.4	0.4
Leucine	0.81	1.78	1.80	1.1	0.7	3.7	0.6
Phenylalanine	2.39	1.84	2.52	1.1	0.5	4.5	0.6
Tryptophan	5.36	1.04	1.44	1.9	0.8	9.8	1.2

* Signal-to-noise ratio = 2.

ing (*e.g.*, ref. 28), so that the minimum detectable sample concentration will be reduced to a smaller extent. We used a 7-μl sample loop with the microbore columns, as against a 25-μl loop with the conventional columns.

Table I summarizes the results of the comparison. Miniaturization of the system reduced the absolute detection limits by a factor 6–11, and the lowest detectable concentrations by a factor 1.6–3.3.

Further reduction of detection limits in both systems can be expected when a pulse-free pump is used, as most of the noise is caused by pulsations in the flow-rate.

An application of the use of a microbore column is shown in Fig. 4. A urine sample was diluted 1:2 with a phosphate buffer solution to give approximately the same composition as the mobile phase, and spiked with phenylalanine and tryptophan. The concentrations ($\pm$ S.D., n = 4) of these amino acids in this particular unspiked urine sample were calculated to be 11.8 $\pm$ 0.9 and 7.2 $\pm$ 0.7 mg l^{-1}, respectively. These values are well within the range normally found for human urine[29].

Separation with borate and carbonate buffers

When a borate buffer was used as mobile phase, the shape of the peaks was strongly influenced by the sample concentration. When the concentration of the amino acids was reduced, the peaks became broader and showed more tailing while the peak maxima appeared somewhat later. The separation of the eight essential

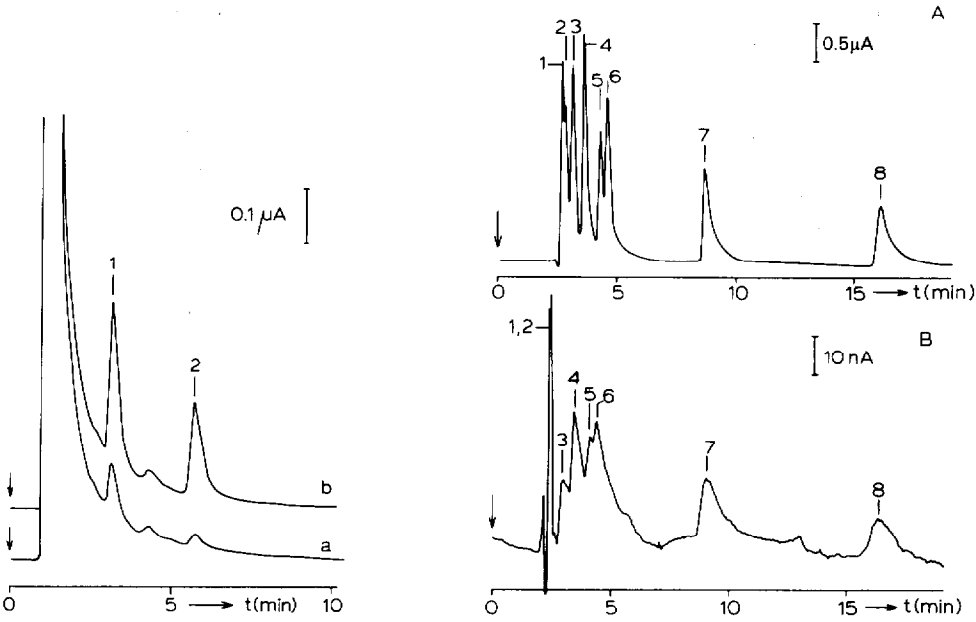

Fig. 4. Determination of phenylalanine and tryptophan in urine. Column: 250 × 1.1 mm I.D., 7.7 μm LiChrosorb RP-18. Mobile phase as in Fig. 1. Urine diluted 1:2: (a) blank; (b) spiked with 10^{-4} *M* phenylalanine (1) and tryptophan (2).

Fig. 5. Separation of essential amino acids with borate buffer. Mobile phase: 0.025 *M* borate buffer, 10% methanol, pH = 7.8. Other conditions as in Fig. 1. Sample concentrations: (A) $5 \cdot 10^{-4}$ *M*; (B) 10^{-5} *M*.

TABLE II

INFLUENCE OF SAMPLE CONCENTRATION ON PEAK WIDTH

Plug injections of 25-μl leucine solutions. Mobile phase: 0.025 *M* borate buffer, 10% methanol, pH 7.8. Flow-rate: 1.0 ml/min.

Amino acid concentration (mol l^{-1})	*Peak width at fraction x of the peak height (sec)*	
	x = 0.6	*x = 0.1*
10^{-2}	4.1	15
10^{-3}	4.7	19
10^{-4}	7.6	30
10^{-5}	10.9	40

amino acids, which was comparable to that obtained with a phosphate mobile phase at a sample concentration of $5 \cdot 10^{-4}$ *M*, was largely lost at a sample concentration of 10^{-5} *M* (see Fig. 5).

The electrode reaction in a borate buffer is known to be slow[24]. To investigate if the slow electrode process was the cause of the deterioration of the chromatograms at low amino acid concentrations, plug injections (without column) were made with 25-μl samples of different concentrations of leucine. Table II shows that the apparent loss of separation is indeed caused by the fact that the electrode response is slow, especially when the current differs only slightly from the baseline value. It may be concluded that the borate system can be used for the analysis of concentrated amino acid samples, but should be avoided in trace analysis.

It has been shown that the separation of amino acids and small peptides on reversed-phase columns can be improved by using a mobile phase with a high pH instead of a neutral buffer solution[30,31]. The stability of bonded phase columns at high pH values is low, but Iskandarani and Pietrzyk[31] have achieved good separations on a polymer-type reversed-phase column material, PRP-1 (Hamilton). We have investigated the use of this material with carbonate buffers (pH 9–11) as the mobile phase.

At these high pH values, all amino acids used are known to react rapidly with copper(II) ions[24]. An improvement in the sensitivity at low flow-rates, as observed for most amino acids with phosphate-containing mobile phases, is therefore not expected in a carbonate system. However, to save on the expensive packing material, we tried to pack microbore columns. Unfortunately, we could not succeed in packing columns with more than a few hundred theoretical plates. Nevertheless, some selected amino acids could be separated adequately (Fig. 6).

Again, increased broadening and tailing of the peaks was observed when the sample concentration was decreased. Consequently, the peak heights for dilute sample solutions were considerably lower than expected in comparison with concentrated samples (curve b in Fig. 7). However, measurements on plug injections (curve a) of phenylalanine solutions showed that the electrode response rate was not causing these phenomena. An explanation must be sought in the presence of a limited number of strong adsorption sites in the stationary phase. This is clearly illustrated by curve c in Fig. 7. Here, a small amount (10^{-4} *M*) of a strongly retained amino acid,

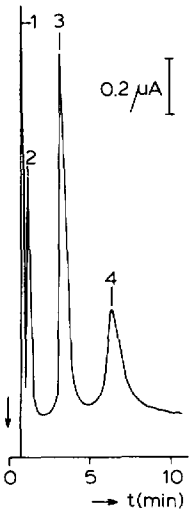

Fig. 6. Separation of amino acids with carbonate buffer. Column: 250 × 1.1 mm I.D., 10 μm PRP-1. Mobile phase: 0.1 *M* carbonate buffer, 10% methanol, pH = 11.0. Flow-rate: 0.23 ml min^{-1}. Sample (7 μl): (1) 0.19 μg of valine; (2) 0.21 μg of leucine; (3) 1.07 μg of phenylalanine; (4) 1.33 μg of tryptophan.

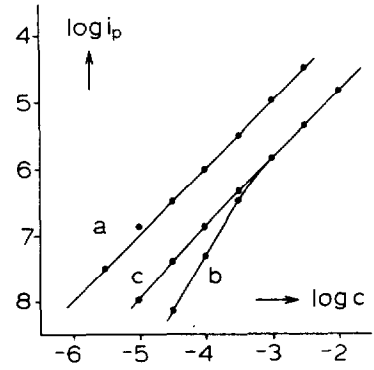

Fig. 7. Linear dynamic range of phenylalanine with carbonate buffer. Conditions as in Fig. 6. (a) Plug injections; (b) with column; (c) as (b) with 10^{-4} *M* tryptophan added to the mobile phase.

tryptophan, was added to the mobile phase. Apparently this amino acid occupies the strong adsorption sites, so that the less retained phenylalanine gives better peaks at low concentration. Unfortunately, this method reduced the stability of the column. The column collapsed after a few hours, and its performance could be restored only by flushing with mobile phase without tryptophan for several hours.

CONCLUSIONS

The predictions based on a study with the rotating disk copper electrode[24] are followed well with a flow-through cell used as an HPLC detector. As the rotating disk system is easier to handle than a dynamic system and quantitative interpretation of the results is theoretically better founded, the use of this electrode as a tool for optimization of an electrochemical HPLC detector can be recommended.

The amperometric detection of amino acids has been shown to be a selective, simple and reliable method. The sensitivity is compared with that of other frequently used methods in Table III. The detection limits are of the same order of magnitude as with absorbance measurement after ninhydrin derivatization. Lower detection limits have been reported for underivatized amino acids with UV absorbance measurements at 200 nm[20]. However, in the analysis of biological fluids considerable interference from other compounds must be expected with this method. The sensitivity of fluorescence measurements was not achieved in our work. However, the advantage of amperometry over fluorimetry is its simplicity and the absence of the need for derivatization. Thus it offers a gain in analysis time (compared with pre-column derivatization for fluorimetry) and cost (compared with post-column derivatization). The data in

TABLE III

COMPARISON OF SENSITIVITY OF DETECTION METHODS FOR AMINO ACIDS IN HPLC

Compounds separated	*Detection method*	*Typical detection limits (pmole)*	*Ref.*
PTH derivatives	UV absorbance	5–50	6, 8, 9, 10
DNP derivatives	UV absorbance	10–50	11
Dansyl derivatives	Fluorimetry	0.05–1	14, 32, 33
OPA derivatives	Fluorimetry	0.05–0.5	16, 17, 34
Amino acids	UV absorbance, 200 nm	1–10	20
Amino acids	Ninhydrin reaction and absorbance	10–100	1, 35
Amino acids	OPA reaction and fluorimetry	0.5–10	36, 37*
Amino acids	Potentiometry with copper electrode	100–500	21, 22
Amino acids	Amperometry with copper electrode	10–100	This work

* Refers to primary amines.

Table III refer to separations on conventional-scale columns. As has been shown in Table I, the use of microbore columns provides, apart from the advantages normally attributed to miniaturization (*e.g.*, ref. 38), some extra gain in sensitivity for the amperometric method.

A drawback to the use of a copper electrode is the fact that only certain neutral or alkaline buffer solutions can be used, while most separations are performed with acidic mobile phases, *e.g.*, in ion-exchange and ion-pair chromatography. This can be overcome by post-column addition of an alkaline solution to an acidic mobile phase. We intend to investigate this method.

Preliminary experiments have indicated that under appropriate conditions the copper electrode responds to other ligands also, *e.g.*, dicarboxylic acids. The detection and separation of these compounds is now under study.

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