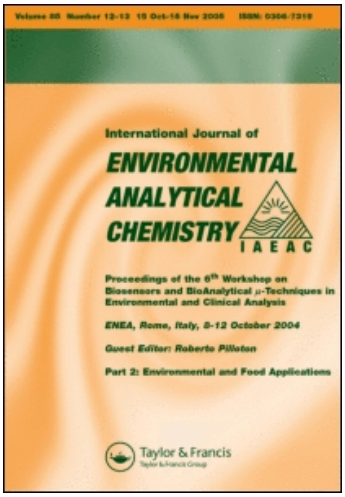

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Application of HPLC in the Determination of Amino Acids in Natural Waters

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Application of HPLC in the Determination of Amino Acids in Natural Waters

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Reversed-phase high performance liquid chromatography (HPLC) with gradient elution has been used for analysing 19 different amino acids at picomole level using fluorimetric detection.[1] The amino acids in natural waters were preconcentrated prior to their analysis. Calcium ions interfere in the determination of amino acids and a way to overcome this effect has been described.

KEY WORDS: Amino acids determination, HPLC, natural waters.

INTRODUCTION

Organic matter in natural waters is found as biopolymers and they account for about 10% of the total dissolved organic carbon (DOC), whereas the amino acids produced by the hydrolysis of biopolymers constitute less than 1% of the DOC.[2] Organic matter forms complexes with trace metals found in natural water and therefore may have impact on the environment.[3] For example, trace amounts of amino acids (ca. 100 μg/l) found in sea water complexes with metal ions, in particular copper ions. Since the complexing properties of amino acids with trace metals are of analytical interest, the determination of amino acids in natural water by HPLC has been investigated in this paper.

Amino acids in natural waters are found at levels below the detection limit and hence they have to be preconcentrated prior to their analysis. A freezing concentration technique[4] was chosen for this purpose because it minimizes the possibility of chemical modification of amino acids.

The direct determination of amino acids by reversed-phase liquid chromatography is not possible because of their polarity and also they cannot be detected by fluorimetry or absorption spectrometry. However, by using a derivatization reaction with dansyl chloride (Dns),[5,6] phenylthiohydantoin (PTH)[7–9] or O-phthaldialdehyde (OPA),[1] the detection limit can be decreased and the resolution improved. Because of the good sensitivity and reproductibility, we have chosen the derivatization by OPA in this study.

Calcium ions are present in natural waters and may interfere in the analysis and hence the effect of calcium in the analysis has also been studied.

EXPERIMENTAL

Preconcentration

A Rotavapor BUCHI was used for freezing concentration. A Cryomat Bath Cooler with methanol was used for lowering the temperature to ca. $-12°C$.

HPLC

A Pye Unicam (LC3 XP-pump) chromatographic system coupled to a microprocessor for gradient elution was used. The solvent was degassed by heating it to 55°C and bubbling helium through the solution. The sample was injected by means of a sample loop, i.e., usually 20 or 200 μl.

A Varian Fluorichrom fluorimeter with 25-μl cell was used as the detector. Excitation and emission wavelengths used were 340 nm (Filter 7–54) and 460 nm (Filter 4–76). The chromatograms were recorded on a two-pen W + W chart recorder.

Columns

The 25 cm × 0.32 cm I.D. analytical column was slurry packed with a

stationary phase RP-C_{18}, of particle size 7 μm (Polygosil 60–7C_{18} of Macherey–Nagel, West Germany). The 4 cm × 0.32 cm I.D. precolumn was also filled with RP-C_{18} but the particle size used was 10 μm.

Reagents

Unless otherwise stated all reagents used were Merck P.A. Acetonitrile (solvent B) and a binary mixture of 12% (v/v) acetonitrile in pH 7.2 phosphate buffer ($1.25 \cdot 10^{-2}$ M Na_2HPO_4) were used as mobile phase (solvent A).

The following calcium salts were used for studying the effect of calcium on amino acid analysis: Calcium chloride, calcium carbonate and calcium nitrate. Suprapur calcium carbonate was heated at 830°C for 24 h to remove traces of organic matter.

All the solutions were prepared with high purity water obtained by running demineralized water through millipore Milli Q system.

Procedure

Phosphate buffer 17.8 g of $Na_2HPO_4 . 12H_2O$ were dissolved in 800 ml water, the pH was adjusted to 7.2 with concentrated HCl and the solution diluted to 1 liter.

Borate buffer. 75 ml of a saturated solution of H_3BO_3 were adjusted to pH 9.5 with NaOH and diluted to 100 ml. For pH 8 and 9 Merck titrisol buffers were used.

Derivatization reagent. 50 mg of o-phthaldialdehyde (OPA) were dissolved in 4.5 ml of CH_3OH. 50 μl of C_2H_5SH and 0.5 ml of the desired buffer (pH 8, 9 or 9.5) were then added.

Amino acids standards. A solution containing all the amino acids at 10^{-6} M was prepared.

Derivatization of amino acids. 0.5 ml of the amino acids solution was added to a mixture of 0.5 ml of buffer (pH 8, 9 or 9.5) and 100 μl of derivatization reagent.

Elution. Gradient elution (change from 12 to 53% of CH_3CN) proceeds as shown in Table I.

TABLE I

Gradient elution program

No.†	Time (min)	% Final B
1	4	16
2	3	17
3	5	18
4	5	24
5	5	26
6	20	53

†Start, 12% B.

RESULTS AND DISCUSSION

Typical results obtained for the separation of 19 different amino acids at picomole level are presented in Table II.

TABLE II

Separation of 19 different amino acids

Peak No.	Amino acid	k'	α	R_s
1	Ac.Asp	1.10		
2	Ac.Glu	2.70	2.45	2.80
3	Aspn	11.40	4.22	8.70
4	Ser	13.80	1.21	2.28
5	Gln	15.10	1.09	1.16
6	His	16.20	1.07	0.86
7+8	Gly+Thr	20.70	1.28	2.90
9	Ala	25.60	1.24	3.50
10	Arg	27.20	1.06	1.39
11	Tyr	29.70	1.09	2.27
12	Val	35.80	1.08	2.37
13	Meth	37.00	1.03	1.29
14	I−Leu	39.20	1.06	2.50
15+16	Leu+Trp	40.60	1.03	1.69
17	Phe	41.40	1.02	0.96
18	Orn	53.20	1.28	14.40
19	Lys	54.40	1.02	1.67

Freezing concentration

The amino acids recovery after concentration was found to be less than 100% and the amount of amino acids lost during this step depends on the concentration factor. The percentage of amino acids recovered can be obtained from the slope of the linear portion of the graph of C_i/C_o vs. V_o/V_i, where C_i and C_o are the final and initial concentration of amino acids, respectively; V_i and V_o are the final and initial volume, respectively.

It was found that as the initial amino acid concentration increases (over the range 5.10^{-7}–2, 5.10^{-5} M), the recovery of amino acids varies between 86%–94% (Fig. 1). Thus the chromatographic method can be applied to samples containing amino acids at levels greater than 10^{-7} M, where 90% recovery of amino acids can be achieved with the freezing concentration method.

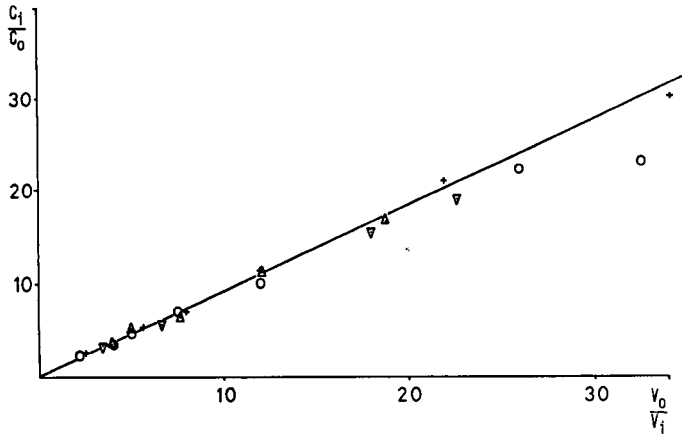

FIGURE 1 Freezing concentration of amino acids having various concentrations: $+5.10^{-7}$ M, ○ 10^{-6} M, Δ 5.10^{-6} M, ∇ 2, 5.10^{-5} M.

Interferences

The presence of calcium in the sample increases the intensity of some of the amino acids peaks. Chromatograms of amino acids in the presence and absence of calcium are shown in Figs. 2–3. A chromatogram of the blank containing calcium is shown in Fig. 4.

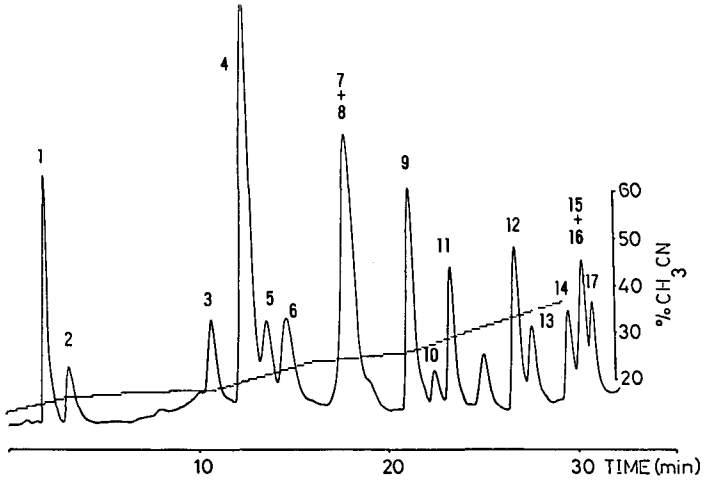

FIGURE 2 Interference of Ca: standard amino acids + 0.02 M Ca^{2+}, pH = 9.5.

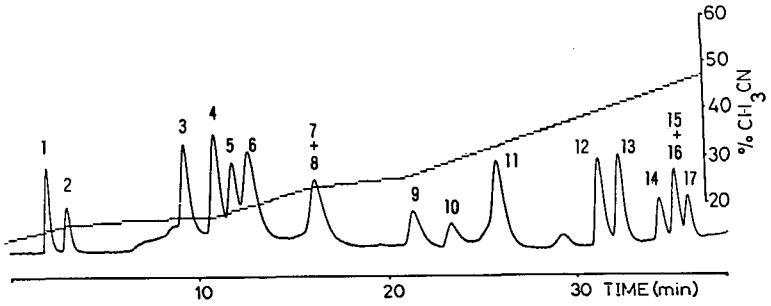

FIGURE 3 Standard amino acids, pH = 8 or 9.5.

The area of the chromatographic peaks in the presence of calcium ions was found to be dependent on the pH and the type of buffer used. The effect of pH on the interference and derivatization of amino acids is summarized in Table III. From the results, it can be seen that over the pH range 8–9 derivatization of amino acids is complete and the interference effects due to calcium ion are eliminated. The effect of interference of calcium will be reported in detail later.

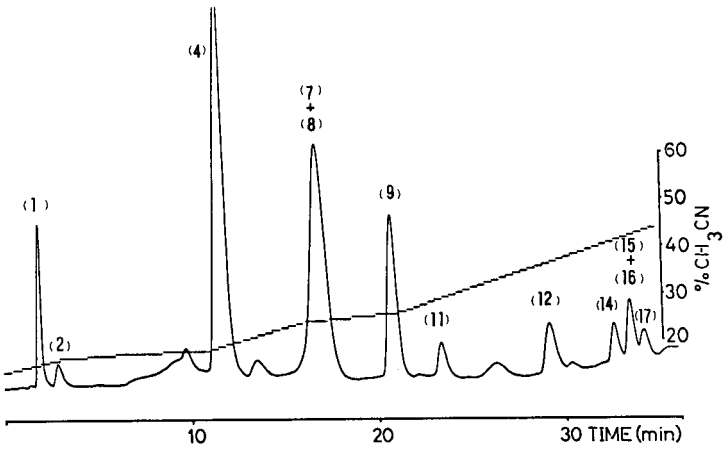

FIGURE 4 Effect of Ca only: OPA/C_2H_5SH + 0.02 M Ca^{2+}; pH = 9.5.

TABLE III

Effect of pH on the Ca(II) interference and derivatization of amino acids

pH	7	8	9	9.5 (HCO_3^-/CO_3^{2-})	9.5 (borate)
Interference from Ca at 0.02 M	nil	nil	nil	weak	strong
Derivatization of amino acids	partial	complete	complete	complete	complete

APPLICATION OF HPLC TO ANALYSIS OF AMINO ACIDS IN NATURAL WATERS

A typical chromatogram of amino acids in natural waters samples is shown in Figure 5. The chromatogram obtained after eliminating interferences due to calcium is shown in Figure 6. A comparison of Figure 5 with Figure 6 shows that by carrying out amino acids determination at pH 8, the effect due to calcium is suppressed. The analyses of three lake waters are shown in Table IV.

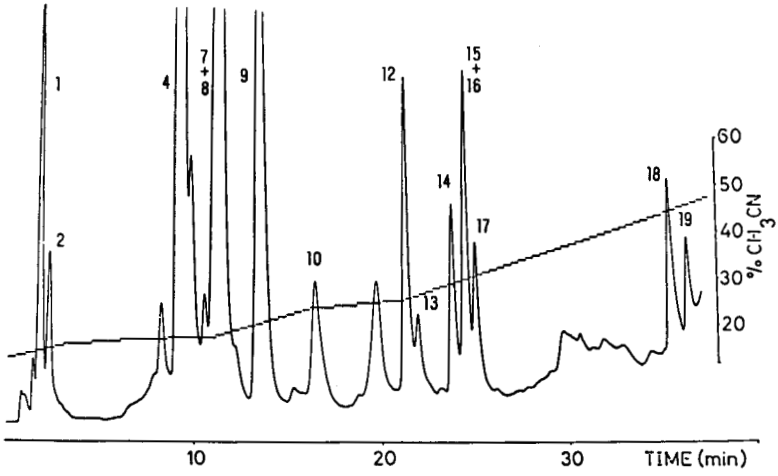

FIGURE 5 Interference of Ca: Amino acids in Lake Geneva (Geneva, Switzerland) after ca. 10-fold preconcentration; pH = 9.5.

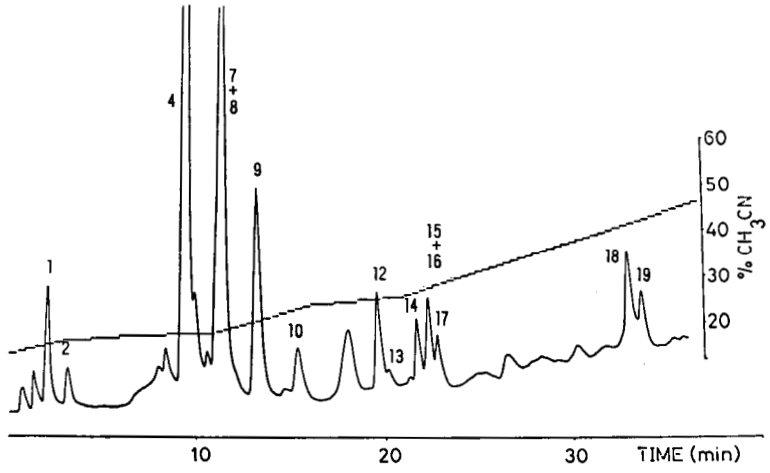

FIGURE 6 Elimination of Ca: Amino acids in Lake Geneva (Geneva, Switzerland) after 10-fold preconcentration; pH = 8.

TABLE IV

Amino acid concentrations found in natural water (10^{-8} M)

Amino acid	Lake Geneva	Lake Bret	River Arve
Ac.Asp	2.82	1.16	2.34
Ac.Glu	0.85	0.72	2.15
Ser	25.22	14.20	24.98
Gly+Thr	26.24	13.62	27.14
Ala	6.25	4.62	9.66
Arg	1.59	1.43	3.36
Val	2.21	0.97	2.41
Meth		0.71	0.77
I−Leu	1.27	0.61	1.16
Leu+Trp	1.62	0.65	1.98
Phe	0.69	0.23	0.86
Orn	1.97	0.94	2.41
Lys	0.58	0.74	1.72

CONCLUSION

The results obtained in this study show that amino acids in natural waters can be determined by reversed-phase HPLC by concentrating water prior to analysis. A concentration factor of 10 to 20 by means of the freezing concentration technique is required for their determination. Using the freezing technique recoveries of about 90% are obtained for the amino acids. If higher concentration factors are required, repetitive freezing concentration is recommended.

The limitation of the freezing technique is that during preconcentration of amino acids, the calcium ions present in the sample are also concentrated and these may interfere in the analysis of amino acids by masking the amino acids peaks. This interference can be readily eliminated by carrying out the derivatization reaction with OPA in the pH range 8–9. Thus HPLC offers a simple and rapid means of analysing amino acids in natural waters.

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