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OPERATIONAL PARAMETERS OF VOLTAMMETRIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETECTORS WITH COPPER ELECTRODES AND APPLICATION TO A DETERMINATION OF SOME FODDER BIOFACTORS

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SUMMARY

Two versions of an amperometric detector with a copper working electrode have been constructed and tested for high-performance liquid chromatography (HPLC). The performance of the detectors was studied using selected amino acids. The dependence of the detector response on the mobile phase flow-rate was studied in the range common in both macro- and microcolumn HPLC (5 μl/min to 1.0 ml/min). It has been found that the detection sensitivity generally increases with decreasing flow-rate, *i.e.*, the detector response is governed by the rate of the complexation reaction between the cupric ions and the solutes. This fact makes amperometric detection with a copper electrode especially useful for microcolumn separations. For all 20 amino acids studied, calibration curve parameters and detection limits have been determined; the latter vary from 0.4 to 18 ng in the injected volume. The amino acids can also be sensitively detected in a medium of 0.1–1.0 *M* ammonia, which is promising for the use of strong anion exchangers in amino acid separations. Choline can also be detected at a copper electrode, with a detection limit of 40 ng. An HPLC method with amperometric detection at a copper electrode has been developed for the determination of lysine, methionine and choline in fodder biofactors.

INTRODUCTION

It has recently been shown by several authors[1–14] that amino acids and a number of other substances can be detected electrochemically by employing complexation reactions of the solutes with cupric ions. This method was initially[1] based on the postcolumn reaction of amino acids with cupric ions added at a constant flow-rate, the loss in cupric ion concentration being monitored potentiometrically with a cupric ion-selective electrode. The main drawback of this system, elution curve broadening

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in the postcolumn reactor, has been removed by the use of a detector with a metallic copper tubular[2,3] or wire[4–10] electrode; the variations in the electrode potential, caused by complexation of the cupric ions present in the oxide layer on the electrode surface, are measured. This method has been applied not only to amino acids[2], but also to other organic acids[4,5], inorganic anions[6,9] and to indirect detection of some inorganic ions[9], including those of the alkaline earth metals[7,8,10]. A general disadvantage of all these potentiometric measurements is non-linearity of the calibration graphs and limited sensitivity, *e.g.*, the detection limits for glycine, valine and isoleucine are 75, 200 and 300 ng/μl, respectively.

Amperometric detection[11–14] has been shown to be more sensitive and versatile. When a metallic copper electrode is polarized at a slightly positive potential, a film of hydrated oxides is formed on its surface, which is associated with the passage of a small, constant anodic current. In the presence of substances that form stable complexes with cupric ions the solubility of the oxide film is enhanced and thus the anodic current increases. A similar effect also occurs with other metals, *e.g.*, mercury, cobalt and nickel[15,16], but these materials are less suitable. The oxidation of mercury produces free mercuric ions that are rapidly transported from the electrode surface, and the surface oxidation of cobalt and nickel is rather slow and complicated. The oxidation of copper is relatively simple and rapid and cupric ions form stable complexes with a number of ligands.

Kok *et al.*[11–13] have demonstrated that this detection of amino acids can be carried out only in neutral and alkaline solution (range pH 6–11) and that phosphate and carbonate buffers are suitable for this purpose, whereas a borate buffer is not because of the slow electrode reaction. The detection sensitivity increases with decreasing buffer concentration, with increasing pH and, for slow complexation reactions, with decreasing mobile phase flow-rate. These authors have also applied the method to the determination of oxalic acid in biological matrices[14].

The limits imposed on the pH range and the mobile phase composition are certainly significant drawbacks of this method. On the other hand, this approach is promising for high-performance liquid chromatographic (HPLC) analyses of many complex-forming compounds in complicated matrices, as it is highly selective. For this reason we have studied in detail the operational parameters of a detector of our own construction using amino acids as solutes, and investigated applications to some substances other than amino acids. The application to a determination of choline, lysine and methionine in fodder biofactors is described; use of this method for some other substances will be described elsewhere.

EXPERIMENTAL

Apparatus

The measurements were carried out on MHPP 20 (Laboratorní Přístroje, Czechoslovakia) and LC-XP (Pye Unicam, U.K.) liquid chromatographs. The columns were packed with Separon SI C-18, 5 μm (a glass microcolumn, 150 mm × 1 mm, Laboratorní Přístroje, column 1) or Partisil ODS, 10 μm (a steel column, 250 mm × 4.6 mm, Pye Unicam, column 2). Two detector cells were used, based on our design of carbon fibre detector[17]. The first cell was identical with the carbon fibre cell[17], only the carbon fibres were replaced by thin copper wires (0.08–0.1 mm in

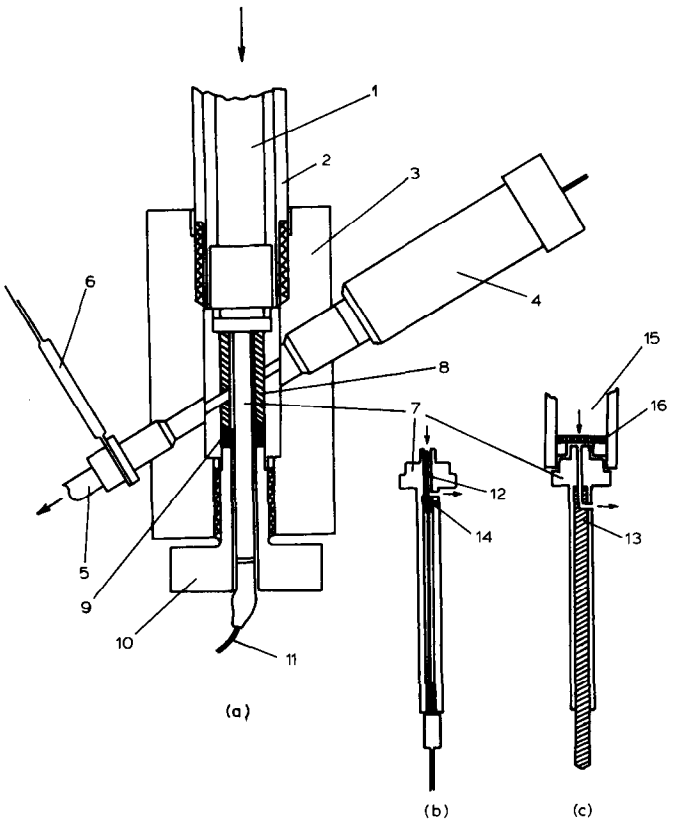

Fig. 1. Voltammetric detector cell. (a) Overall view; (b) working electrode 1 (a strand of copper wires, 0.08–0.1 mm in diameter); (c) working electrode 2 (a copper rod with a channel 0.5 mm in diameter and 3 mm long). 1 = Glass column; 2 = metal mantle; 3 = detector plastic body; 4 = reference electrode (a saturated silver/silver chloride electrode); 5 = stainless-steel outlet tube serving as the counter electrode; 6 = lead to the counter electrode; 7 = PTFE tube containing the working electrode; 8 = porous ceramic tube; 9 = silicone rubber seal; 10 = plastic screw closing the working space; 11 = lead to the working electrode; 12 = copper wires; 13 = copper rod; 14 = silicone rubber seal; 15 = column packing; 16 = metallic frit.

diameter, detector 1). The second cell is depicted in Fig. 1; it contains a tubular copper electrode (diameter 0.5 mm, length 3.0 mm, detector 2) and was directly screwed onto the metallic mantle of the glass microcolumn. The internal volumes were 0.2 and 0.6 μl for the former and the latter cells, respectively. The geometry of these detector cells is considerably different from the one used by Kok *et al.*[12], who used a wall-jet system with a copper disk electrode, 3 mm in diameter. An EDLC electrochemical detector (Laboratorní Přístroje) was used. Samples were injected through a 0.5-μl loop (the microcolumn) and a 20-μl loop (the macrocolumn).

Chemicals

The L-amino acids were tyrosine, tryptophan, lysine, methionine, proline, hydroxyproline, aspartic acid, histidine, serine, aspartate (all from Reanal, Hungary),

leucine, valine, threonine, alanine, glutamic acid, glycine, glutamine, arginine, cysteine (all from Lachema, Czechoslovakia), phenylalanine and isoleucine (Calbiochem, Los Angeles, CA, U.S.A.). Choline chloride, SUKL 5614/74-50 was obtained from Medical Supplies (Prague). The mobile phases were prepared from p.a. chemicals (Lachema) which were not further purified.

Procedure

The mobile phases used were 0.025 *M* aqueous potassium dihydrogen phosphate–methanol pH 6.8 and aqueous solutions of ammonia (0.1, 0.5 and 1.0 *M*) and were always degassed by passage of helium. The copper working electrode was activated before measurement by the procedure described in ref. 12, *i.e.*, by polarization for 5 min at −0.3 V, followed by polarization for 15 min at +0.15 V, in the flowing mobile phase. The measurement potential, if not stated otherwise, was +0.15 V. All measurements were carried out at laboratory temperature (22± 2°C) and the potentials are referred to the saturated Ag/AgCl electrode.

RESULTS AND DISCUSSION

Applied potential

Hydrodynamic voltammograms were obtained in the detector cells for glycine, valine and isoleucine in the mobile phase containing 0.025 *M* potassium dihydrogen phosphate with 10% methanol at a flow-rate of 0.3 ml/min, from −0.1 to +0.5 V. At potentials more negative than −0.1 V the oxidation of the copper electrode surface is negligible and thus the formation of amino acid complexes is not perceptible;

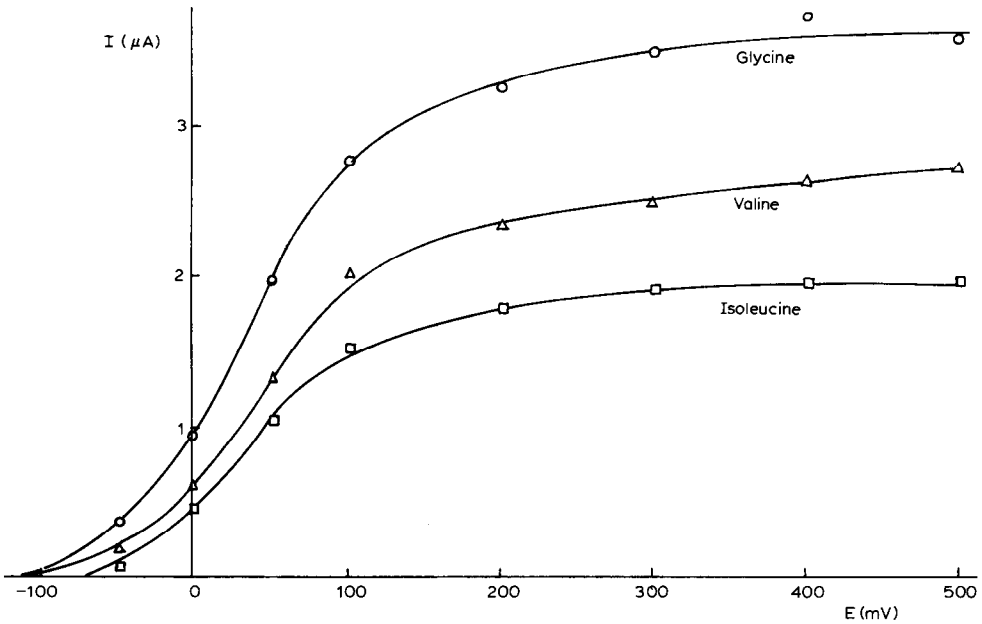

Fig. 2. Hydrodynamic voltammograms of glycine, valine and isoleucine in 0.025 *M* aqueous KH_2PO_4 + 10% methanol. Flow-rate: 0.3 ml/min. Detector 1, column 2. The background current is subtracted.

at potentials more positive than +0.5 V the copper oxidation is so rapid that the large background current can no longer be compensated. The voltammograms corrected for the background current (Fig. 2) are similar for all three substances, indicating an optimum applied potential of +0.15 V.

Flow-rate

The dependences of the detector response on the mobile phase flow-rate were studied for histidine, glycine, valine and isoleucine at +0.15 V and under the same conditions as above, from 0.1 to 1.0 ml/min (Fig. 3). For histidine, this dependence was also measured in the flow-rate region common in microcolumn chromatography, *i.e.*, from 0.005 to 0.1 ml/min. The response always decreased with increasing flow-rate; the decrease was approximately linear for glycine and exponential for the other amino acids, being most pronounced for histidine. This suggests that the detector response is controlled entirely by the kinetics of the complexation reaction. These results are somewhat different from those of Kok *et al.*[12] who found that the response to rapidly reacting amino acids (histidine) increased with increasing flow-rate, *i.e.*, was at least partially controlled by convective diffusion of the solute.

Response time and response volume

The dependences of the response time (the time required for the signal to attain 63.2% of the maximum value upon a step change in the solute concentration) and the response volume (the response time multiplied by the flow-rate) were studied

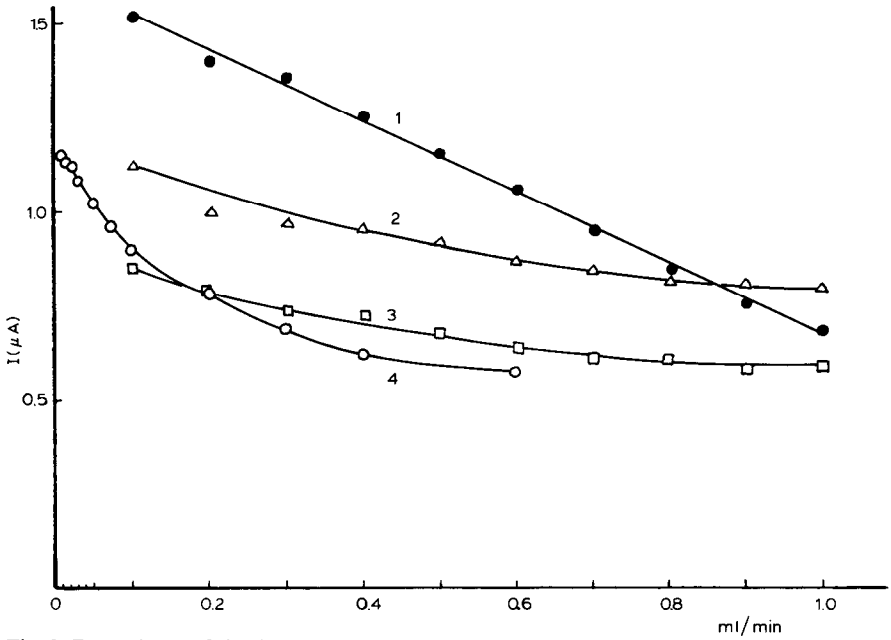

Fig. 3. Dependence of the detector response on the mobile phase flow-rate. Curves: 1, glycine; 2, valine; 3, isoleucine; 4, histidine. Applied potential; +0.15 V. Other conditions as in Fig. 2 (for flow-rates below 0.1 ml/min; column 1, detector 2).

TABLE I

DEPENDENCE OF THE RESPONSE TIME AND RESPONSE VOLUME ON THE MOBILE PHASE FLOW-RATE

Without column, detector 1. Applied potential: +0.15 V. Mobile phase: 0.025 *M* KH_2PO_4 + 10% methanol.

Amino acid	*0.2 ml/min*		*0.5 ml/min*		*0.8 ml/min*		*1.0 ml/min*	
	Response time (s)	*Response volume (μl)*	*Response time (s)*	*Response volume (μl)*	*Response time (s)*	*Response volume (μl)*	*Response time (s)*	*Response volume (μl)*
Glycine	14.6	48.7	8.6	71.6	6.0	79.9	4.2	69.9
Valine	15.5	51.7	8.6	71.6	6.4	85.3	4.8	79.9
Isoleucine	15.9	53.0	8.1	67.5	6.0	79.9	5.0	83.3

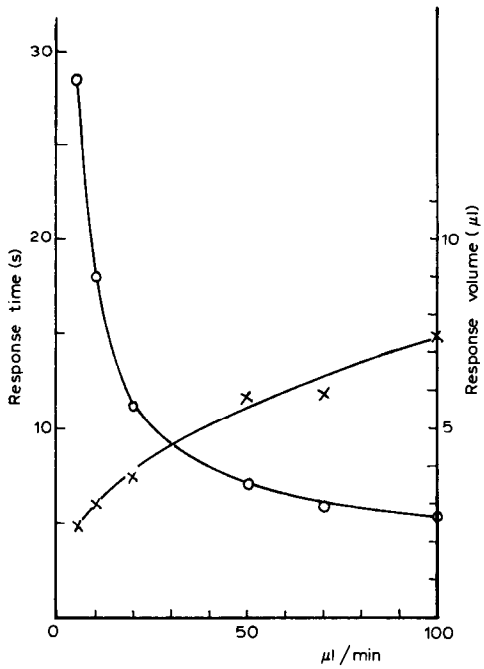

Fig. 4. Dependence of the response time (○) and the response volume (×) on the mobile phase flow-rate for histidine. Detector 2, column 1. Applied potential: +0.15 V. Mobile phase: 0.025 *M* KH_2PO_4 + 10% methanol.

under the same conditions as above, but with direct injection of glycine, valine, isoleucine and histidine into the detector. The results are given in Table I and in Fig. 4. It is seen that with increasing flow-rate the response times decrease and the response volumes increase, but these dependences are not very pronounced except at the lowest flow-rates (see Fig. 4). At higher flow-rates, the response times are considerably longer than those obtained with this type of detector with direct electrochemical reaction of the solute at the working electrode (*cf.*, ref. 17). The relatively large response volumes also indicate a considerable effect of the kinetics of the chemical reaction involved.

Another indication of a decisive effect of the kinetics of the complexation reaction on the detector response is the fact that the same results were obtained with detectors that differ in their hydrodynamics. This holds even for the amino acids, *e.g.*, histidine which react most rapidly.

Calibration curves

The calibration parameters for all the studied amino acids, obtained under the same conditions as above, at a flow-rate of 0.3 ml/min and an applied potential of +0.15 V, are summarized in Table II. The calibration graphs exhibit good linearity up to solute amounts of *ca.* 200 ng. Some amino acids, such as histidine, serine, arginine and glutamic acid, can be detected in amounts of the order of tenths of a nanogram, whereas the detection limits for the other amino acids amount to a few nanograms. It follows from the above results that this detection will be especially

TABLE II

CALIBRATION CURVE PARAMETERS

Column 2, detector 1. Mobile phase: 0.025 *M* KH_2PO_4 + 10% methanol; flow-rate 0.3 ml/min. Applied potential: +0.15 V. Samples: 20 μl.

Amino acid	*Correlation coeff.*	*Slope of the regression line (μA/μg)*	*Detection limit (ng)**
Tryptophan	0.9985	8.2	8.0
Valine	0.9961	11.8	4.1
Phenylalanine	0.9969	12.6	1.9
Tyrosine	0.9996	7.6	4.9
Alanine	0.9975	6.9	9.0
Methionine	0.9999	10.6	4.5
Proline	0.9930	2.5	18.0
Isoleucine	0.9974	5.3	12.8
Aspartic acid	0.9887	5.4	13.0
Glycine	0.9999	16.4	1.7
Lysine	0.9997	9.6	6.7
Glutamine	0.9988	9.6	4.8
Aspartate	0.9992	5.7	10.5
Histidine	0.9989	57.4	0.4
Serine	0.9938	52.3	0.5
Arginine	0.9997	36.2	0.8
Cysteine	0.9804	17.7	1.6
Threonine	0.9930	29.9	1.1
Glutamic acid	0.9997	54.8	0.6
Leucine	0.9959	17.5	1.6

* The detection limit is twice the peak-to-peak noise.

useful with microcolums. A successful separation of six amino acids on the microcolumn used is depicted in Fig. 5.

A number of other substances yield a signal at a copper electrode. Our experiments have demonstrated that, *e.g.*, choline can be detected with a relatively high

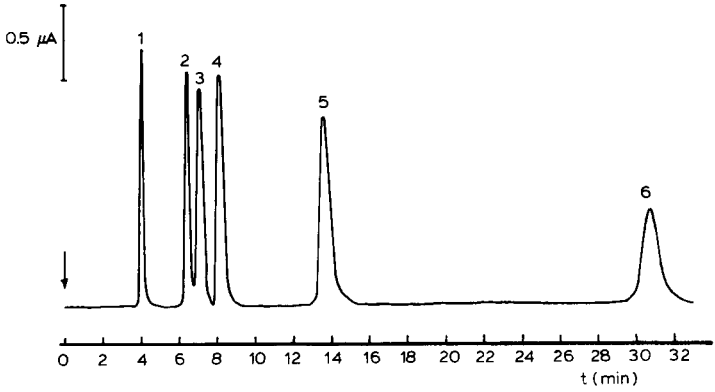

Fig. 5. Separation of glycine (1, 100 ng), methionine (2, 130 ng), isoleucine (3, 250 ng), tyrosine (4, 190 ng), phenylalanine (5, 105 ng) and tryptophan (6, 115 ng) on a glass microcolumn (1) with detector 2. Flow-rate: 20 μl/min. Mobile phase: 0.025 *M* KH_2PO_4, pH 6.7. Applied potential: +0.15 V. A sample of 0.5 μl of the mixture was injected at laboratory temperature.

TABLE III

DETECTION LIMITS OF SEVERAL AMINO ACIDS AT VARIOUS AMMONIA CONCENTRATIONS

Ammonia concentration (M)	*Potential (V)*	*Detection limit (ng)*			
		Histidine	*Methionine*	*Lysine*	*Hydroxyproline*
0.1	−0.1	1.5	4.8	6.0	3.7
0.1	+0.1	2.5	3.7	2.8	3.4
0.5	−0.3	4.8	4.1	4.5	6.8
1.0	−0.3	3.0	2.1	1.3	3.4
1.0	−0.4	–	–	1.6	–

sensitivity. The calibration graph is linear up to an injected amount of 75 μg and the detection limit is 40 ng (correlation coefficient 0.9997, slope of calibration graph 1.23 μA/μg).

Ammoniacal mobile phases

In view of the possibility of using strong anion exchangers for separation of amino acids, detection in aqueous solutions of ammonia was also studied. The detector response was measured for histidine, methionine, lysine and hydroxyproline in the following aqueous mobile phases: (a) 0.1 *M* NH_3, pH 10.66; (b) 0.5 *M* NH_3, pH 11.10; (c) 1.0 *M* NH_3, pH 11.28. The amino acids yielded a sensitive response in all three media, which was only slightly dependent on the ammonia concentration (apparently due to small changes in the pH). The detection limits are listed in Table III.

The residual current increases strongly with increasing ammonia concentration. Whereas in 0.1 *M* ammonia it is possible to employ copper electrode potentials from +0.1 to −0.4 V at a high measurement sensitivity, in 0.5 *M* ammonia the sensitivity must be substantially decreased and in 1 *M* ammonia only negative potentials of −0.3 to −0.4 V can be used in order to be able to compensate the high background current (at potentials of −0.5 V and higher no detector response is obtained). In 0.5 and 1.0 *M* ammonia, the amino acids yield a negative response on an high background. These phenomena are due to the competition from ammonia as a ligand for Cu^{2+}: stability constants[18] for $Cu(NH_3)_x^{2+}$ are $\log \beta_1 = 4.13$; $\log \beta_2 = 7.61$; $\log \beta_3 = 10.48$ and $\log \beta_4 = 12.59$.

These results indicate that the use of anion exchangers with aqueous ammoniacal mobile phases and voltammetric detection at a copper electrode is promising for the determination of amino acids, as the serious problem involved in the use of chemically bonded phases, *i.e.*, the discrepancy between the pH values required for the separation (pH 2–3) and the detection (pH $\geqslant 6$), is removed.

Determination of lysine, methionine and choline in fodder biofactors

Biofactors added to animal fodder mixtures contain various amino acids, mainly lysine and methionine, specifically effective substances, vitamins, protective substances, biocatalysts, growth stimulators, inorganic substances and other components. Thus the matrix is very complicated and analytical control is highly impor-

TABLE IV

DETERMINATION OF CHOLINE, LYSINE AND METHIONINE IN FODDER BIOFACTORS

The codes specify the kind of animal and its weight.

Code	*Active component*	*Theoretical value (mg/g)*	*Determined value (mg/g)*	
			Electrochem.	*Spectrophot.*
418 KZK	Choline	100	130	132
419 KZK	Choline	100	116	119
K	Choline	120	130	128
PCH	Lysine	100	85	
P1	Lysine	80	82	
COS	Lysine	50	47.5	
BR2	Methionine	50	45	

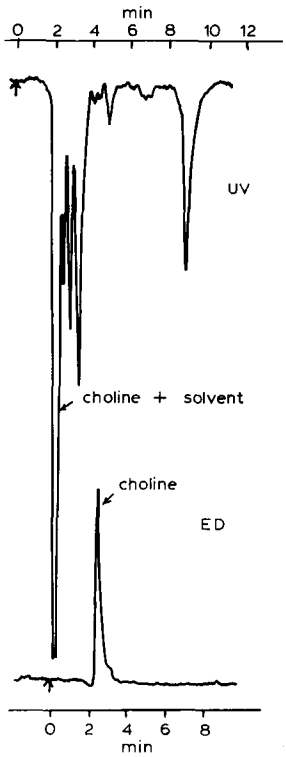

Fig. 6. Determination of choline in the 418 KZK fodder biofactor by use of UV photometric and electrochemical detection. Column 2; detector 1. Mobile phase: 0.025 *M* KH_2PO_4 + 10% methanol. Injected amount: 10 μl (25 mg dissolved in 10 ml of the mobile phase). Electrochemical detection: +0.15 V; sensitivity, 20 nA/cm; recorder range, 1 V. UV photometric detection: 254 nm; sensitivity, 0.04 a.u.f.s.; recorder range, 20 mV.

tant for veterinary reasons. HPLC with electrochemical detection employing a copper electrode is advantageous for the determination of the amino acids present and of choline, in view of the high selectivity of the method. The results of analyses of selected biofactors are given in Table IV. A comparison of the UV photometric and electrochemical methods of detection is shown in Fig. 6. It is evident that the UV photometric signal of choline is indistinguishable from the signals of other components, whereas the signal of the voltammetric detector with a copper electrode is selective for choline.

CONCLUSION

The amperometric detection with a copper electrode permits a sensitive determination of amino acids and of a number of other compounds forming complexes with copper ions. The main limitation of the detection method is the necessity of working at a pH greater than 6, which is outside the optimum range for reversed-phase separation. This problem can be overcome either by a postcolumn change in the pH, which is disadvantageous from the point of view of elution curve broadening, or by using ion-exchange stationary phases (see the above results for ammonia mobile phases). The detection selectivity can be utilized to advantage in analyses of samples with complicated matrices. The results for choline obtained by amperometric detection are in a good agreement with the spectrophotometric determination using ammonium reineckate (see Table IV). The precision of the method (relative standard deviation $\pm 2\%$, obtained for five parallel measurements) is satisfactory. The voltammetric detection at a copper electrode is especially advantageous for microcolumn HPLC, high sensitivity being attained at very low mobile phase flow-rates.

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