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DETERMINATION OF OXALIC ACID IN BIOLOGICAL MATRICES BY LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

Oxalic acid was separated from interfering compounds with a solvent-generated ion-exchange chromatographic system, and detected with a copper electrode amperometric detector. The lower limit of detection in standard solutions was 5 ng. Complex samples, such as vegetable extracts and urine, could be analysed with a minimum of sample preparation.

INTRODUCTION

Many studies have been devoted to the determination of oxalic acid in biological matrices. The measurement of the oxalic acid content of food-stuffs, such as rhubarb, may be important in breeding programmes or for dietary reasons. The oxalate concentration of body fluids is an indicator for various bodily disorders, of which the growth of kidney stones is the most widely known.

Though the literature on methods for the determination of oxalic acid in various samples is extensive (see *e.g.*, ref. 1), this determination is still an object of research. This is due to the relatively poor detectability of oxalate, which necessitates a rigorous sample clean-up. Sample preparation often involves precipitation of oxalate as its calcium salt, a method easily leading to large systematic errors or low reproducibility. Even for methods involving chromatographic separation the sample preparation must often be extensive. For instance, using high-performance liquid chromatography (HPLC) with UV detection at 220 nm, even for the determination of the high oxalate content in rhubarb a clean-up step is necessary[2]. More selective detection methods with HPLC have been used, such as pre-column derivatization[3] and oxidative electrochemical detection[4,5], but these methods are still not very satisfactory for routine analyses of body fluids.

In previous papers[6,7] we have reported on the use of an amperometric detector for HPLC based on a copper working electrode for the selective detection of compounds rapidly complexing with copper(II) ions. The detector has been applied for amino acid analyses[8]. Polycarboxylic acids are also known to form complexes with copper(II) ions. In this work we have studied the feasibility of the copper electrode

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detector for the determination of oxalic acid in biological matrices. Since appreciable retention for oxalic acid cannot be expected in conventional reversed-phase HPLC, and since ion-exchange columns still fail to reach the efficiency of bonded-phase columns, our separation study has focused on the ion-interaction reversed-phase chromatography of oxalate ion.

EXPERIMENTAL

Instrumentation

Batch measurements were carried out with a rotating copper disk electrode of 6-mm diameter, driven by a servomotor with tachogenerator. The chromatographic equipment consisted of a Waters 6000 A pump delivering a flow of 1 ml/min, a Rheodyne injection valve with a 20-μl loop, 100 × 4.6 mm columns packed in the laboratory with LiChrosorb RP-8 (10 μm) and RP-18 (7 μm) (Merck, Darmstadt, F.R.G.) and Spherisorb Amino phase (5 μm) (Phase Sep, Queensferry, U.K.) and a Metrohm 1096/2 detector cell with a copper working electrode (3-mm diameter), a glassy carbon auxiliary electrode and a Ag/AgCl/1 *M* LiCl reference electrode. The working electrode potential was kept at 0.1 V with a potentiostat/amplifier made in the laboratory. All measurements were performed at ambient temperature.

Reagents and solutions

The carboxylic acids used, of analytical or reagent-grade purity, were obtained from various sources. Tetrabutylammonium perchlorate (TBA) and tetrahexylammonium iodide (THA) were obtained from Eastman (Rochester, NY, U.S.A.). THA was converted into the nitrate form by titration with silver nitrate and filtering. Hexadecyltrimethylammonium bromide (cetrimide) was obtained from Baker (Deventer, the Netherlands).

Solutions were prepared with doubly distilled demineralized water and Baker grade methanol. Other chemicals used were of analytical-grade purity.

Standard solutions of carboxylic acids were prepared shortly before use from aqueous 0.05 *M* stock solutions.

The mobile phase consisted of an aqueous phosphate buffer of pH 7.0 and 10% (v/v) methanol, and was degassed by purging with nitrogen at 30°C.

Sample preparation

Vegetable samples were blended in a ten-fold excess of water, the slurry was diluted 1:10 with 1 *M* hydrochloric acid, a drop of *n*-octanol was added, and the mixture was heated to 100°C for 15 min. After cooling, the mixture was filtered and the filtrate was used for injection either directly or after dilution with mobile phase solution.

Urine samples were filtered and used for injection either directly or after dilution with mobile phase solution.

RESULTS AND DISCUSSION

Electrode response

Voltammograms with the rotating (1200 rpm) copper disk electrode were re-

TABLE I

COPPER ELECTRODE RESPONSE TOWARDS ORGANIC ACIDS RELATIVE TO PHENYLALANINE

Compound	*Relative response*
Phenylalanine	1
Histidine	3.6
Oxalic acid	1.17
Malonic acid	0.22
Succinic acid	0.00
Phthalic acid	0.00
Maleic acid	0.00
Malic acid	0.40
Tartaric acid	0.35
Citric acid	4.7
Isocitric acid	0.36

corded in 10% (v/v) methanol containing phosphate buffer, and the limiting current increase after addition of a mono-, di- or tricarboxylic acid was measured. For comparison two amino acids were also tested: histidine, which reacts very rapidly with copper ions, and phenylalanine, which exemplifies the majority of naturally occurring amino acids with respect to reaction rate[6]. None of the monocarboxylic acids (acetic, glycolic, lactic, salicylic, mandelic) investigated caused an increase of the limiting current. In Table I the electrode responses of the di- and tricarboxylic acids tested are given, relative to that of phenylalanine. Large differences in response are observed which cannot be explained completely by differences in acid dissociation and/or complexation constants as given in the literature[9]. However, it must be kept in mind that the electrode response is governed by complexation reaction kinetics rather than by equilibrium conditions. Structural effects may play a much more important role, as is illustrated by the response difference between maleic and malic acid, which are both fully deprotonated at pH 7 and for which the equilibrium constants of the copper(II) complexes have about the same value. From Table I it can be seen that the copper electrode detector is very selective, which may be disadvantageous if one is interested in an "acid profile" of a sample, but advantageous for the determination of one or two compounds in a complex matrix.

Chromatography

At first, separation of oxalic acid from interfering acids was pursued by ion-pairing chromatography on a LiChrosorb RP-18 column with TBA or THA salts added to the mobile phase. The influence of the concentration of ion-pairing reagent to the retention is shown in Fig. 1. Although the dicarboxylic acids could easily be separated from the tricarboxylic, their mutual resolution was poor. The retention could be influenced by changing the ionic strength or the methanol content of the mobile phase, but conditions under which oxalic acid was completely separated from malic, malonic and tartaric acid were not found with TBA or THA reagents. The same trend was found with the amino column, which in addition suffered from a low efficiency. Lowering the mobile phase pH to take advantage of the differences in acidity constants is not possible with the copper electrode detector, which has to be operated using neutral or basic buffer solutions.

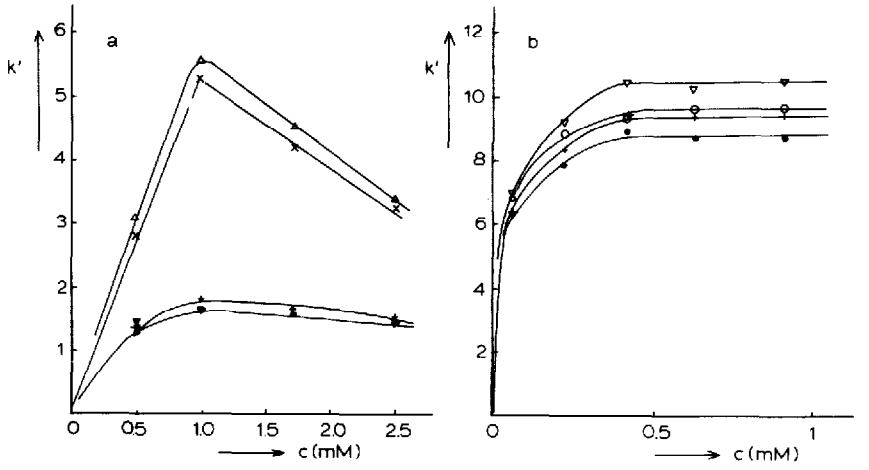

Fig. 1. Influence of the ion-pairing reagent concentration on the retention of oxalic (○), malic (+), malonic (●), tartaric (▽), citric (×) and isocitric (△) acid. Column: 100 × 4.6 mm I.D., 7 μm, LiChrosorb RP-18. Mobile phase: 10% methanol in aqueous 0.01 *M* phosphate buffer, pH 7. Ion-pairing reagent: (a) TBA, (b) THA.

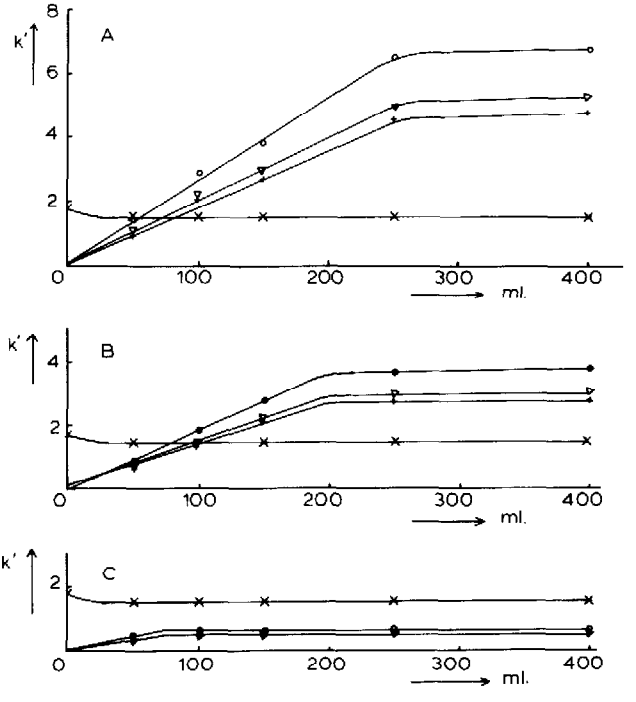

Fig. 2. Influence of the volume of a $5 \cdot 10^{-4}$ *M* cetrimide solution flushed over the column on the retention of oxalic (○), malic (+) and tartaric (▽) acid and phenylalanine (×). Methanol content of the cetrimide solution: (A) 25%, (B) 35%, (C) 50%. Column packed with 10 μm LiChrosorb RP-8. Mobile phase: 10% methanol in aqueous 0.025 *M* phosphate buffer, pH 7.

As an alternative, we tried a solvent-generated or dynamic anion-exchange separation system[10]. A hydrophobic stationary phase can be loaded with the cationic surfactant cetrimide by flushing it which a cetrimide-containing water–organic modifier mixture. Once the column is loaded, cetrimide can be left out of the mobile phase. This is fortunate, since clogging of capillaries by cetrimide and electrode poisoning by bromide are thus avoided.

A LiChrosorb RP-8 column was loaded with cetrimide dissolved in various water–methanol mixtures ($5 \cdot 10^{-4}$ *M*). The retention of the carboxylic acid on the column, measured with aqueous 0.025 *M* phosphate buffer containing 10% methanol as the mobile phase, increased with the volume of cetrimide solution flushed over the column until a saturation level was reached (Fig. 2). Clearly, when this saturation is not yet reached, the column is loaded only partially. This is also evident from the column plate number, which is substantially lower at non-complete saturation, reflecting cross-sectional differences in the length of column packing covered with cetrimide. Still, even with complete loading the column efficiency is considerably and irreversibly decreased compared with that of the original alkyl-modified silica column.

The amount of cetrimide adsorbed on the column and the retention times of the carboxylic acids could be decreased by increasing the methanol content of the cetrimide solution with which the column was loaded. Retention times on a particular loaded column were reproducible, within 1.5–2.5%, and a column could be used for months without significant bleeding of cetrimide from the column.

On a column loaded with a solution of $5 \cdot 10^{-4}$ *M* cetrimide in 25% methanol, oxalic and malic acid were separated from malonic and tartaric acid, which eluted

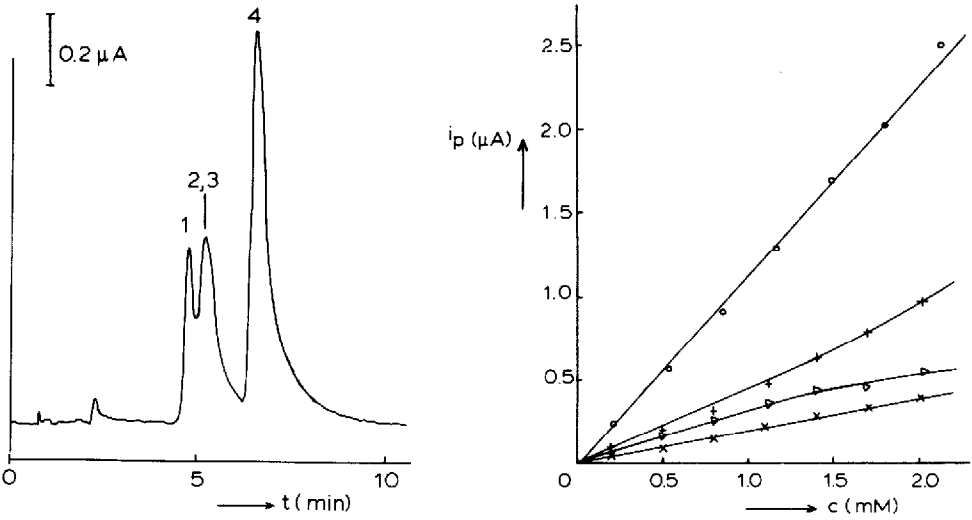

Fig. 3. Chromatogram of a standard mixture of (1) malic, (2) malonic, (3) tartaric and (4) oxalic acid. Column loaded with $5 \cdot 10^{-4}$ *M* cetrimide solution in 25% (v/v) methanol. For further conditions see Fig. 2.

Fig. 4. Calibration plots for oxalic (○), malic (+), malonic (×) and tartaric (▽) acid. For conditions see Fig. 3.

together (Fig. 3). Citric and isocitric acid were retained very strongly (k' greater than 50).

The detector sensitivity for the various acids is approximately as expected from the batch studies. Calibration plots are linear for oxalic and malonic acids up to $2 \cdot 10^{-3}$ *M*, for malic and tartaric acid deviations from linearity were observed above a concentration of $1 \cdot 10^{-3}$ *M* (Fig. 4). Lower limits of detection, calculated for a signal-to-noise ratio of 2 with an observed noise of 1.5 nA, vary from 5 ng for oxalic to 30 ng for malonic acid. Peak height reproducibility for standard solutions was within 3% when measured on the same day. Day-to-day variance was larger (10–20%); the sensitivity had to be redetermined, especially after polishing of the electrode, which was done every few days.

Application to real samples

To study the recovery of oxalic acid during the preparation of vegetable samples, part of a sample was spiked directly after blending. The peaks in a chromatogram of a real sample are broader than those of a standard solution. This is probably caused by the high ionic strength of the former solutions. A high ionic strength decreases retention, so that the sample will be smeared out over the first part of the column before they are diluted by the mobile phase. Therefore, recoveries were calculated from peak areas instead of peak heights. The broadening of the peaks was highly reproducible, so that later on peak heights could be used again for the calculation of concentrations with a correction coefficient. The results of the recovery study are given in Table II. The amounts of oxalic acid found in vegetables are of the same order of magnitude as those reported by others[2].

From the data in Table II on urine samples it can be seen that the absence of sample clean-up does not lead to electrode poisoning. The sensitivity (calculated from peak areas) for (added) oxalate in urine is the same as in standard solutions. The relative peak-area variation of four consecutive urine injections was 3.6%. The concentrations measured in urine are in agreement with the normal values of 14–50 mg/l (ref. 11).

The main interfering compound, especially in urine samples, is the late eluting citric acid. Five or six samples could be analyzed for oxalic acid before the first citric acid peak appeared. Instead of waiting for the citric acid peaks to elute, which would

TABLE II

RECOVERY OF OXALIC ACID FROM BIOLOGICAL SAMPLES

Sample	*Oxalic acid added*	*Oxalic acid found*		*Recovery (%)*
		Spiked	*Blank*	
Rhubarb, A	8.3 mg/g	15.9 mg/g	7.4 mg/g	102
Rhubarb, B	8.5	12.1	3.3	104
Rhubarb compote	5.2	8.3	2.9	104
Spinach	8.3	16.6	7.3	112
Urine, A	36 mg/l	70 mg/l	36 mg/l	95
Urine, B	–	–	15	–
Urine, C	45	72	28	102

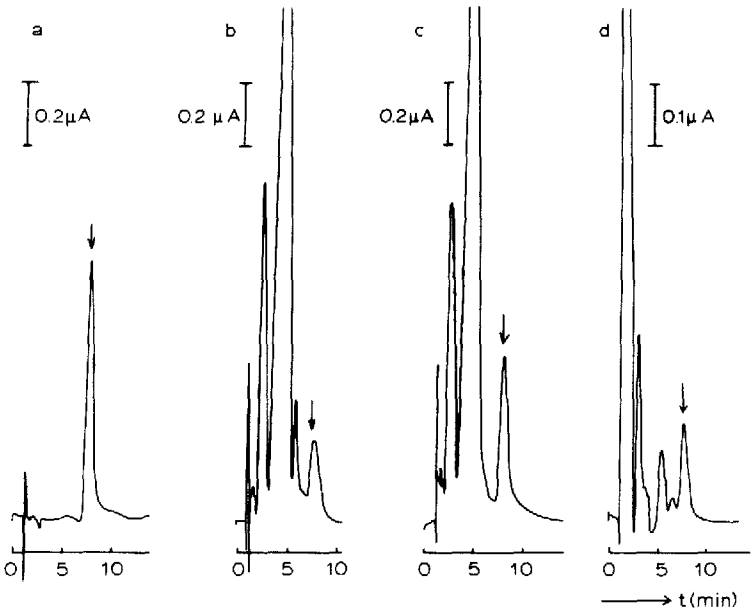

Fig. 5. Chromatograms of (a) standard, (b) rhubarb, (c) spinach and (d) urine samples. The arrow indicates the oxalic acid peak. For conditions see Fig. 3.

double the mean time of analysis, the procedure could be speeded up by flushing the column with a few millilitres of phosphate buffer (pH 2). This approach did not influence column performance. During the column cleaning procedure the detector was turned off to prevent extensive copper electrode abrasion.

Some typical chromatograms of real samples are shown in Fig. 5.

CONCLUSIONS

The combination of solvent-generated ion-exchange chromatography with a highly selective electrochemical detection system allows the determination of oxalic acid in complex matrices with a minimum of sample preparation. The method compares successfully with previously published chromatographic methods, which involve tedious sample preparation procedures[3,4] or show insufficient selectivity[5]. The sensitivity of the detector is amply adequate for analysis of, *e.g.*, urine samples.

The copper electrode detector can also be used for the determination of citric acid. In that case we recommend an ion-pairing separation system with TBA added to the mobile phase (Fig. 1a).

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