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Quantification of intermediates involved in the cyclic 2,3-diphosphoglycerate metabolism of methanogenic bacteria by ion-exchange chromatography

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ABSTRACT

A novel method was developed to quantify the intermediates involved in cyclic 2,3-diphosphoglycerate metabolism, *i.e.* cyclic 2,3-diphosphoglycerate, 2,3-bisphosphoglycerate, 2-phosphoglycerate, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate and phosphate. The method consists of an ion-chromatographic separation followed by conductivity and ultraviolet detection. In a single run (41 min) all compounds were readily resolved, except for 2- and 3-phosphoglycerate. Concentrations down to 10–20 μM and up to 1.0–1.5 mM can be accurately determined. The method was suitable for the analysis of cell-free extract and for the determination of enzymic conversions of the compounds involved. It was found to be reliable and faster than isotachophoresis or enzymic determination.

INTRODUCTION

Cyclic 2,3-diphosphoglycerate (cDPG) is a 2,3-bisphosphoglycerate (2,3-BPG) derivative in which both phosphate groups are connected by a phosphoric anhydride binding [1–3]. The occurrence of cDPG in nature is restricted to methanogenic bacteria belonging to the genera *Methanobacterium, Methanobrevibacter, Methanothermus, Methanosarcina* and *Methanosphaera* [4–6]. In these organisms the compound may be present in concentrations from 2 mM to as high as 1.5 M [3,7–10]. The function of cDPG is not completely clear, and the compound is suggested to act in storage of energy [1,11,12], phosphorus [2] or cell carbon [7,13]. As the *in vivo* counter ion of potassium, cDPG may also play a role in thermostabilization of proteins in extreme thermophilic organisms [4]. cDPG is synthesized and degraded as shown in Fig. 1 [12,14].

To come to a better understanding of cDPG metabolism and its potential regulation, suitable analysis techniques are required for cDPG and its cellular precursors and metabolites. After acid hydrolysis of cDPG into 2,3-BPG, the compound may be analysed enzymically [3]. Enzymic methods are also

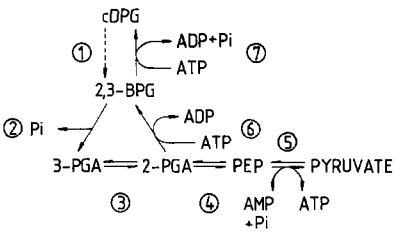

Fig. 1. cDPG biosynthesis and degradation related on the intermediate cell carbon (C_3) metabolism in *M. thermoautotrophicum* [12]. The numbers refer to the following enzymes: 1 = cyclic 2,3-diphosphoglycerate hydrolase; 2 = 2,3-biphosphoglycerate phosphatase; 3 = phosphoglycerate mutase; 4 = enolase; 5 = phosphoenolpyruvate synthetase; 6 = 2-phosphoglycerate kinase; 7 = cyclic 2,3-diphosphoglycerate synthetase.

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available for the other compounds shown in Fig. 1. However, the methods are laborious and time-consuming; each component requires a separate analysis. A number of these drawbacks may be overcome by isotachophoresis [11,12], which enables the simultaneous analysis of the relevant compounds. This technique, however, still may be slow and is tedious to handle. As an alternative, this paper describes the use of ion-exchange chromatography connected to a background suppressor system for the identification and quantification of the compounds involved in the cDPG metabolism.

EXPERIMENTAL

Chemicals

ATP was purchased from Boehringer (Mannheim, Germany). N-tris(hydroxymethyl)methyl-2-aminoethanesulphonate] (TES), 2,3-bisphosphoglycerate (2,3-BPG), 2-phosphoglycerate (2-PGA), 3-phosphoglycerate (3-PGA) and phosphoenolpyruvate (PEP) were obtained from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane] (Tris), potassium chloride, magnesium chloride, oxalate and pyruvate were from Merck (Darmstadt, Germany). Dipotassium hydrogenphosphate, sodium hydroxide (analysed reagent) and sulphuric acid (instra-analysed reagent) were purchased from J. T. Baker (Deventer, Netherlands). cDPG was purified as described previously [11]. The concentration was determined after acid hydrolysis into 2,3-BPG and subsequent enzymic analysis [3]. Gases were from Hoek-Loos (Schiedam, Netherlands) and were made oxygen-free by passage over a prereduced BASF R3-11 catalyst at 150°C or a BASF R0-20 catalyst at ambient temperature for hydrogen-free and hydrogen-containing gases, respectively. The catalysts were a gift from BASF (Ludwigshafen, Germany).

Ion-exchange chromatography

Separation and quantification of cDPG, 2,3-BPG, 2-PGA (and 3-PGA), PEP, pyruvate and inorganic orthophosphate (P_i) was performed with an HP 1050 Ti automated gradient system consisting of a gradient pump, an autosampler, a UV detector and an analog-to-digital converter (interface 35900), which translated the signal of the pulsed electrochemical detector (conductivity mode; Dionex) to the computer. The system was controlled with a high-performance liquid chromatographic (HPLC) ChemStation (DOS series).

Samples (20 μl) were injected by means of an autosampler on an Ionpac AG5A-5μ guard column (Dionex P/N 037134; 50 mm × 4 mm I.D.) connected to an Ionpac AS5A-5μ anion-exchange analytical column (Dionex P/N 037131; 250 mm × 4 mm I.D.). The column material (capacity, 35 μequiv.) contains an alkanol quaternary amine functional group linked to a latex (polyacrylate) matrix. Elution was performed at ambient temperature; the elution rate was 1 ml/min. Separation took place by a linear gradient in 23 min of 0.75 m*M* sodium hydroxide (pH 10.67) to 120.75 m*M* sodium hydroxide (pH 13.07). Thereafter, sodium hydroxide was kept at 120.75 m*M* for 5 min. Following a 0.1-min gradient to 0.75 m*M* sodium hydroxide and a 13-min equilibration at 0.75 m*M* sodium hydroxide, the next sample could be injected. Eluents were passed through an anion trap column (ATC-1, P/N 037151) placed before the injector to remove carbonate from the buffer. The compounds to be analysed were detected with a Dionex conductivity detector set at 30 μS full scale and with a UV detector set at a wavelength of 215 nm. The conductivity caused by OH^- was suppressed by means of an anion micromembrane suppressor (AMMS, P/N 038019) with a 25 m*M* sulphuric acid regenerant set at a flow-rate of 20 ml/min.

Standard solutions in the range of 0.05–1.5 m*M* were prepared in water and contained 0.5 m*M* oxalate as an internal standard. Samples of 20 μl were injected on the column. Calibration graphs were constructed by plotting the area against the amount (external standard) or by plotting the area response ratio against the amount ratio (internal standard).

Organism and enzyme preparation

Methanobacterium thermoautotrophicum (strain ΔH) (DSM 1053) was cultured on a defined medium [15] under 80% hydrogen–20% carbon dioxide in a 300-l fermenter as documented previously [16]. Cells were harvested under nitrogen with a Sharples continuous centrifuge and stored at −70°C. The preparation of cell-free extract was undertaken under strictly anaerobic conditions as described previously [12]. The dry weight of cell-free extracts was 76 mg/ml. A cofactor-free extract was obtained by

ultracentrifugation of a cell-free extract (36 ml) at 135 000 *g* for 1 h at 4°C. The pellet was washed with anoxic 100 m*M* TES/K^+ buffer (pH 7) and the first and the second supernatants were combined, extensively washed on a Amicon PM-30 filter (cut-off 30 000 dalton) with 100 m*M* TES/K^+ buffer and added to the pellet fraction.

Protein was determined with the Bio-Rad method using γ-globulin as a standard.

Cyclic 2,3-diphosphoglycerate synthetase

The activity of cyclic 2,3-diphosphoglycerate synthetase was determined in 50 m*M* Tris (pH 7). Reaction mixtures (200 μl) prepared in 10-ml serum vials placed inside an anaerobic glove box contained 18 m*M* magnesium chloride, 20 m*M* ATP, 0.5 *M* potassium chloride, 10 m*M* 2,3-BPG and 50 μl of cofactor-free extract (2.07 mg of protein); incubations took place under a hydrogen atmosphere. The reactions were started by placing the vials in a

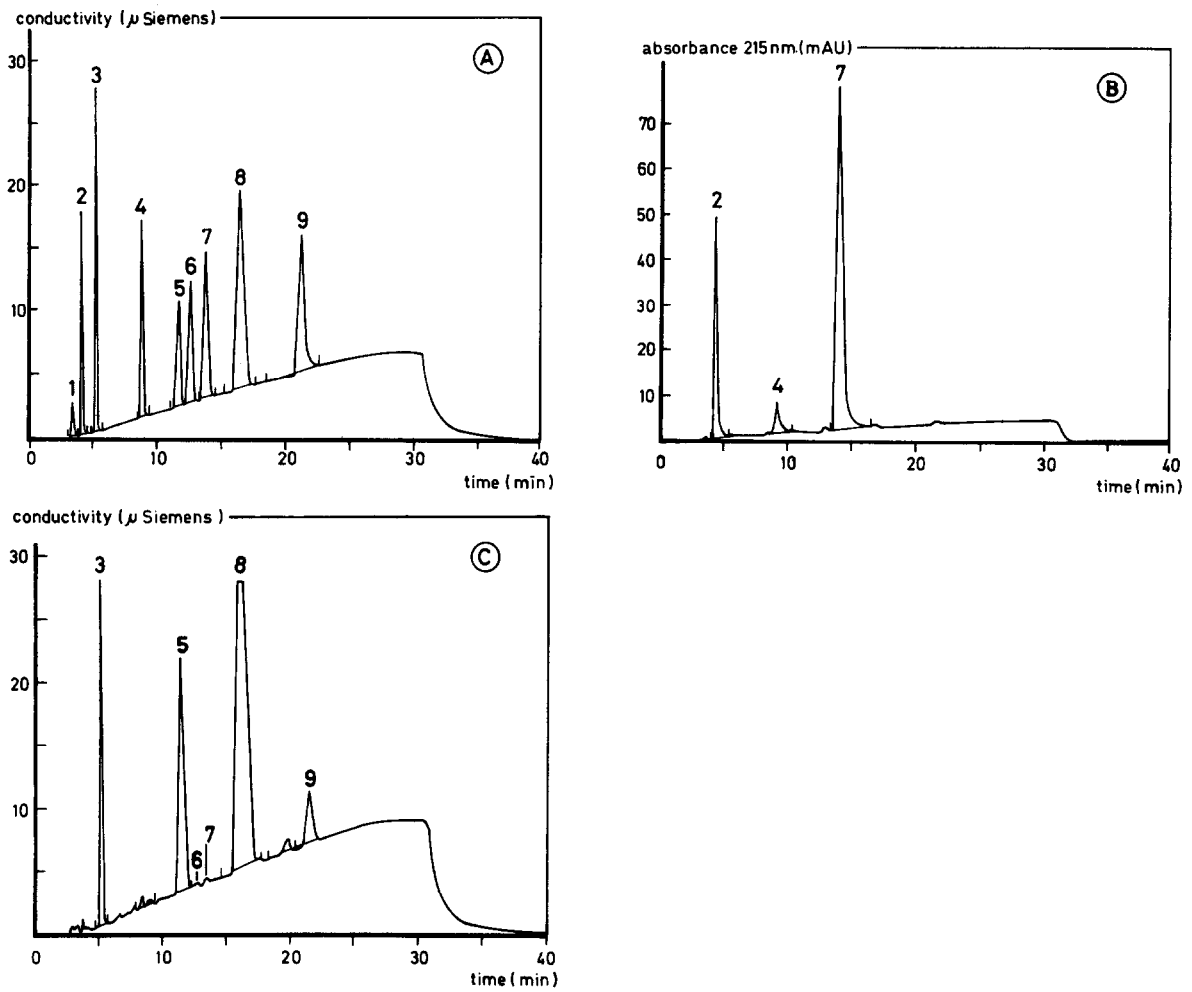


Fig. 2. Ion chromatograms of a standard mixture (A and B) and of a cell-free extract of *M. thermoautotrophicum* (C). Separation was performed as described in the Experimental section; detection took place by conductivity (A and C) or by UV (215 nm) absorption (B). Peaks: 1 = acetate; 2 = pyruvate; 3 = chloride; 4 = oxalate; 5 = phosphate; 6 = 2-phosphoglycerate; 7 = phosphoenolpyruvate; 8 = cyclic 2,3-diphosphoglycerate; 9 = 2,3-bisphosphoglycerate. Standards were present at a concentration of 0.5 m*M* (20 μl injection volume) except chloride and acetate. Cell-free extract was diluted 40-fold and heat-denatured as described in the Experimental section. No internal standard was added in C.

waterbath at 60°C. After an appropriate incubation period the reaction was stopped by placing the vials on ice. Thereafter, the vials were opened, diluted with 750 μl of Milli-Q water and boiled for 30 min. After 10 min of cooling on ice, 50 μl of oxalate (10 m*M*) were added as internal standard and the samples were centrifuged at 12 000 *g* for 10 min at 4°C to remove the denaturated protein. Aliquots of 20 μl of the supernatant were used for analysis on ion-exchange chromatography as described above.

RESULTS

Ion chromatography

The HPLC chromatogram of a mixture of pure compounds obtained by anion-exchange chromatography and conductivity detection is illustrated in Fig. 2A. Pyruvate, oxalate and PEP could also be detected by UV absorption at 215 nm (Fig. 2B). As expected for an anion exchanger, retention of the individual compounds tended to increase with increasing charge of the anions at the pH (10.67–12.04) used during the separation. With the exception of 2-PGA and 3-PGA, the compounds of interest were readily resolved. The retention times of the individual compounds were rather constant, though they decreased somewhat when increasing concentrations were injected. This effect is more pronounced for compounds with higher retention times. Calibration graphs obtained by conductivity and UV detection are shown in Fig. 3A and B, respectively. In Fig. 3C the response and concentration ratios with respect to the internal standard (oxalate) are plotted. Linear regression coefficients of the standard curves were ⩾ 0.999 in Fig. 3A and B and varied between 0.993 and 0.999 in Fig. 3C. Hence, up to at least 20–30 nmol of the various compounds can be accurately determined. The sensitivity of the individual compounds towards conductivity detection increased with increasing negative charge; however, 3-PGA and cDPG are exceptions to this (Fig. 2). Detection limits were of the order of 1–2 nmol, which equals 50–100 μ*M* in the sample when 20 μl are injected. Since the injection volume can be varied from 1 to 100 μl, concentrations down to 10–20 μ*M* may be quantified. Retention times and the (relative) response factors are compiled in Table I.

Analysis of cell-free extract of Methanobacterium thermoautotrophicum

Fig. 2C shows the ion-chromatographic conduc-

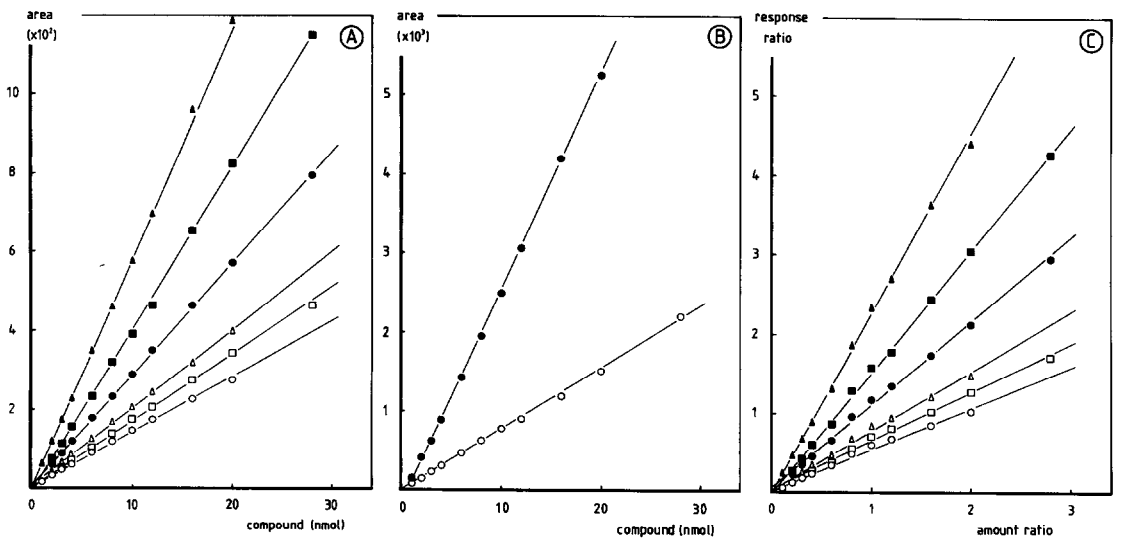


Fig. 3. Calibration graphs of standard compounds detected by conductivity (A) and UV absorption (B). Standards include (○) pyruvate, (□) phosphate, (△) 2-phosphoglycerate, (●) phosphoenolpyruvate, (■) 2,3-BPG and (▲) cDPG. In (C) the response ratio of the compounds with respect to the internal standard (10 nmol of oxalate) is plotted against the amount ratio; detection took place by conductivity. Solid lines are linear regression curves.

TABLE I

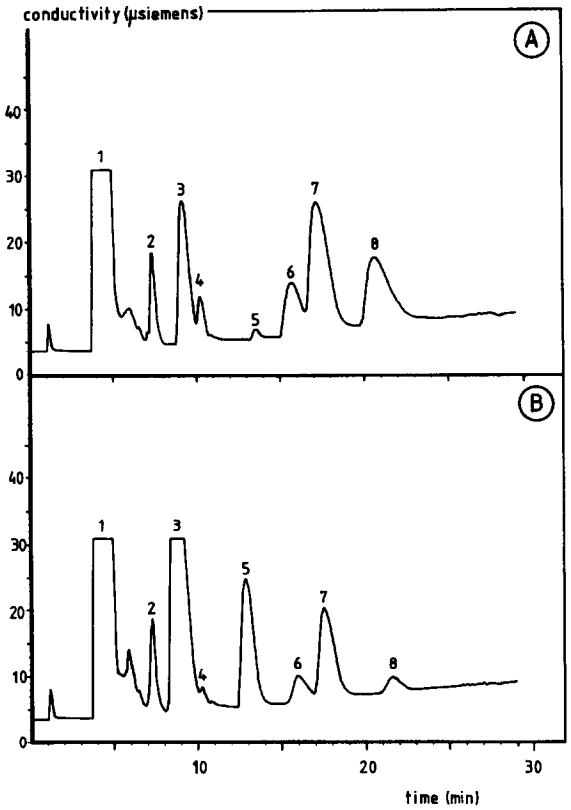
HPLC CHARACTERIZATION OF INTERMEDIATES INVOLVED IN THE cDPG METABOLISM SEPARATED ON AN IONPAC AS5A-5μ ANION-EXCHANGE COLUMN

N.D. = Not determined.

Compound[a]	Retention time (min)	Response factor[b]
Pyruvate	4.00–4.01	0.072 (1.92)
	4.36	0.013
Oxalate	8.71	N.D. (–)
	9.13	N.D.
Phosphate	11.33–11.80	0.060 (1.61)
3-PGA	12.29–12.71	0.036 (0.92)
2-PGA	12.30–12.73	0.050 (1.35)
PEP	13.34–13.97	0.035 (0.95)
	13.75–14.37	0.0036
cDPG	16.03–16.80	0.017 (0.45)
2,3-BPG	20.67–21.61	0.024 (0.65)

[a] The compounds were measured by conductivity detection; a second set of data refers to UV (215 nm) detection.

[b] Response factor is the reciprocal of the linear regression coefficients of the standard curves shown in Fig. 3A and B. The values between parentheses represent the relative response factor, *viz.* the reciprocal of the linear regression coefficients of the curves shown in Fig. 3C.



tivity elution profile of cell-free extract from *M. thermoautotrophicum*. The presence of chloride, phosphate, cDPG and 2,3-BPG, identified on the basis of their retention times (Fig. 2A), is evident. None of these or other compounds were detected by UV absorption (not shown). The amounts of phosphate, cDPG and 2,3-BPG were determined to be 398, 306 and 36.3 μmol/g dry weight, respectively. If one assumes an intracellular volume of the cells of 1.8 ml per g dry weigth of cells [17], the internal concentrations of phosphate, cDPG and 2,3-BPG amount to 221, 170 and 20 m*M*, respectively. The last two values correspond nicely to 170 m*M* cDPG and 22 m*M* 2,3-BPG previously measured by isotachophoretic analysis [11].

Enzymic conversion of 2,3-BPG

To test the application of the method, the ATP-dependent synthesis of cDPG from 2,3-BPG was investigated with a cofactor-free extract of *M. thermoautotrophicum*. Reaction mixtures were prepared and analysed as described in the Experimental section; the results are shown in Fig. 4. From Fig. 4C it

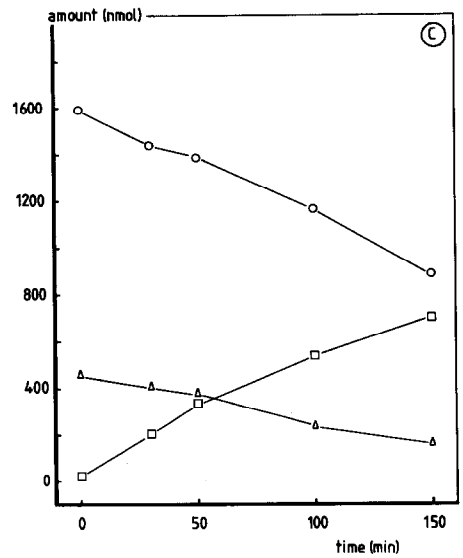


Fig. 4. Conversion of 2,3-BPG. The elution profiles at 0 min (A) and 150 min of incubation (B) are shown. The numbers correspond to chloride (1), oxalate (2), phosphate (3), phosphoglycerate (4), cDPG (5), ADP (6), 2,3-BPG (7) and ATP (8). In (C) the amount of (○) 2,3-BPG, (△) PGA and (□) cDPG are plotted against time.

can be calculated that starting from t = 0 min 2,3-DPG decreased at a rate of 4.6 nmol/min (2.2 nmol/min · mg protein). Concomitantly, cDPG was produced at a rate of 4.5 nmol/min (2.2 nmol/min · mg protein). The amounts of 2,3-BPG converted equalled the amounts of cDPG produced. No cDPG was formed when ATP was omitted (not shown). From Fig. 4A and B it can be seen that ADP and phosphate were produced at the expense of ATP. This conversion, however, cannot be solely attributed to cDPG synthesis, since ATP was also converted in the absence of 2,3-BPG owing to the presence of ATPase and adenylate kinase activities in the crude enzyme preparations. In addition, at t = 0 min about 400 nmol of PGA and 1600 nmol of 2,3-BPG were present (reaction mixtures were prepared with 2000 nmol of 2,3-BPG); thereafter, PGA slowly decreased. These events also took place when either 2,3-BPG or PGA was incubated in the absence of ATP (not shown) and are the result of 2,3-BPG and PGA phosphatase activities in the extract. None of the above reactions were observed when cell extract was omitted from the assays.

The example shows that the HPLC method developed in this paper may be used for analysis of reaction mixtures that contain high concentrations of proteins (10 mg/ml) and where complex reactions simultaneously take place.

DISCUSSION

Ion-exchange chromatography can be used for the determination of anionic compounds. Separation occurs on the basis of charge and conformation and a compound can be identified by its retention time in the chromatogram. We used this technique to quantify the phosphate-containing metabolites present in methanogenic bacteria, *i.e.* cDPG, 2,3-BPG, P_i, 2-PGA, 3-PGA, PEP and pyruvate. With the exception of 2-PGA and 3-PGA, these compounds are readily resolved and may be simultaneously analysed within a single run. Quantification of cDPG and 2,3-BPG in a cell-free extract of *M. thermoautotrophicum* yielded values that were in good agreement with the literature [11]. Application of the method described was demonstrated by the enzymic ATP-dependent conversion of 2,3-BPG into cDPG. The specific activity of cyclic 2,3-diphosphoglycerate synthetase in cell-free extract of *M. thermoautotrophicum* amounted to 2.2 nmol/min · mg protein, which is about ten-fold lower than in *Methanothermus fervidus* [14]. The sensitivity and accuracy of ion-exchange chromatography are of the same order or even better than to enzymic determination [3] or isotachophoretic analysis [12] of the compounds of interest. Enzymic analysis suffers from the disadvantage that each compound must be determined separately and therefore may be elaborate and time-consuming. By isotachophoresis the various compounds also can be measured simultaneously [12]. However, separation of the individual compounds is easily disturbed and a cleaning operation is required every three or four runs. Moreover, automation has not yet been realized. A definite advantage of ion-exchange chromatography is that it is automated, which decreases the operating time: analyses can be performed 24 h a day.

ACKNOWLEDGEMENTS

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