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ISOTACHOPHORETIC ANALYSIS OF ORGANIC ACIDS PRODUCED BY FERMENTATION FROM *n*-ALKANES*

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SUMMARY

Krebs cycle acids formed by fermentation from *n*-alkanes (C_{10}–C_{18}) were evaluated by isotachophoresis. The identity of the acids was established from a conductivity detector for both untreated and electrochemically reduced samples. A full resolution of the acids present in the fermentation media was achieved when separations according to pK values or to complex formation with Ca^{2+} were performed. The results of the determinations of citric and isocitric acids (the most abundant acids) in these separation modes, were in good agreement, with reproducibilities of 1–2%.

INTRODUCTION

Recently, *n*-alkanes have been used as substitutes for the traditional saccharidic substrates in fermentation production of citric acid on an industrial scale. No matter which of these substrates is used, citric acid present in the fermentation medium or in the crude product is always accompanied by other organic acids. For example, under our experimental conditions (see below) isocitric acid was the second most abundant acid, the contents of other Krebs cycle acids being slightly lower.

The optimization and control of this process require rapid and reliable analytical method(s). A variety of methods is available for the determination of citric acid[1], several of which are suitable for fermentation media[2]. Isocitric acid in these media is

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almost exclusively determined by one of the enzymatic methods[2–6]. However, the analysis becomes too complex when simultaneous determinations of these and/or other Krebs cycle acids are required.

Gas chromatography (GC) is a possible technique for simultaneous multicomponent quantitation of these constituents, *e.g.*, refs. 7–9. However, an attempt to elaborate such a method suitable for routine quantitative analysis of the Krebs cycle acids in the fermentation media as well as in the crude citric acid did not give satisfactory results without sample pre-treatment and derivative steps[10].

Isotachophoresis (ITP) in a narrow-bore tube has already been shown to be an excellent method for the analysis of organic acids (for a review see, *e.g.*, ref. 11). Analysis of the Krebs cycle acids was also studied by this technique[12]. A full resolution of a group of the acids was achieved when they were separated according to their pK values. However, such a mode of separation may not be optimal in all instances. Therefore, several other operating systems were tested in this work.

Identification of the constituents in ITP is usually derived from a detector(s) response(s) after their separation(s) in a suitable operating system(s). The results obtained are then compared to those in model experiments. In some instances, constituents of analytical interest are added to the sample (spiking) and the results obtained are compared to those for a pure sample. In respect of the small amounts of the pure components present in the resolved zones and the great flexibility in changing the operating conditions, this technique provides a practical approach to identification.

When preliminary data concerning the qualitative composition of a sample are scarce then the identification cannot be done in such a straightforward way. In this case a well defined chemical or electrochemical sample pre-treatment monitored by ITP, micropreparative ITP[13–16] followed by a post-column identification procedure[14,15,17,18] and a combination of several separation and identification techniques[16] can facilitate identification. We have employed this type of method here, combining an electrochemical sample pre-treatment with ITP.

No other quantitative analytical method giving acceptable results for the present group of constituents is available. Therefore, only ITP determinations were carried out in this work. It is assumed that the different effects defining the effective mobilities of the components in different operating systems makes parallel determinations of the same constituents in at least two systems partly comparable to determinations performed by independent techniques.

EXPERIMENTAL

n-Alkanes (C_{10}–C_{18}) in a mineral nutrient medium were used as substrate for production of citric acid by the microorganism *Candida lypolytica* C-106. A two-stage aerobic cultivation process comprising culture reproduction (48 h) followed by citric acid production was carried out in a Microferm 105 laboratory fermentor (New Brunswick Scientific, Edison, NJ, U.S.A.).

ITP analyses were carried out in an instrument similar to that described by Everaerts *et al.*[11], and provided with a conductivity detection cell designed by Stankoviansky *et al.*[19]. A fluorinated ethylene–propylene copolymer (FEP) capillary tube (20 cm × 0.3 mm I.D.) was used.

Electroreduction experiments were performed in an instrument for electrosynthesis on a microscale developed by Zelenská[20].

Chemicals used for the preparation of the leading and terminating electrolytes were obtained from Sigma (St. Louis, MO, U.S.A.) and from Lachema (Brno, Czechoslovakia). 0.2% Hydroxyethylcellulose (Polysciences, Warrington, PA, U.S.A.) was added to the leading electrolyte. The other chemicals were supplied by Lachema, Sigma, Reanal (Budapest, Hungary), Fluka (Buchs, Switzerland), Calbiochem-Behring (La Jolla, CA, U.S.A.) and Koch-Light (Colnbrook, Great Britain).

RESULTS AND DISCUSSION

Identification

No relevant data concerning the anionic compositions of the fermentation media after the production stage (for the substrate or for the production microorganism) were found in the literature. Therefore, identification was carried out as follows.

(i) A model mixture of the Krebs cycle acids was found which gave isotachopherograms identical to those of fermentation samples in all the operating systems used (Table I). The operating systems were chosen with the aim of differentiating the acids according to their pK values[11] (No. 1), ionic mobilities[11] (No. 3), charges[21] (No. 4) and stability constants[22] (No. 5). The largest numbers of simultaneously identical

TABLE I

OPERATING SYSTEMS

BALA = β-Alanine; EACA = ε-aminocaproic acid; HIS = histidine; BTP = 1,3-bis[tris(hydroxymethyl)methylamino]propane; HEC = hydroxyethylcellulose; ACET = acetic acid; CAPR = caproic acid; MES = 2-morpholinoethanesulphonic acid.

Parameter	*System No.*				
	1	*2*	*3*	*4*	*5*
Leading anion	Cl^-	Cl^-	Cl^-	Cl^-	Cl^-
Concentration (M)	0.01	0.01	0.01	0.01	0.01
Counter ion	BALA	EACA	HIS	BTP	HIS
Co-counter ion	–	–	–	–	Ca^{2+}
Concentration (M)	–	–	–	–	0.002
Additive to the	HEC	HEC	HEC	HEC	HEC
leading electrolyte (%)	0.2	0.2	0.2	0.2	0.2
pH of the leading electrolyte	3.0	4.5	6.0	6.0	6.0
Terminating anion	ACET	CAPR	MES	MES	MES

resolved zones were found in systems 1 and 5. As no contradictory data were found for systems 2–4, we can assume that a full resolution of the anionic constituents present in the fermentation media as well as in the crude citric acid was achieved at pH 3.0 (separation according to pK values) and at pH 6.0 in the complexing system. Citric, isocitric, malic, α-ketoglutaric, *trans*- and *cis*-aconitic acids were identified in these comparative experiments.

(ii) Three of the above acids are electrochemically reducible to acids having

different effective mobilities under identical operating conditions. *Trans-* and *cis*-aconitic acids are reduced to 1,2,3-propanetricarboxylic acid, while α-ketoglutaric acid can be reduced to α-hydroxyglutaric acid. These products of the electroreduction of single acids at a mercury pool electrode were confirmed by ITP. No cross-reactions were detected by ITP when a model mixture of the acids, similar in composition to actual samples, was reduced under identical conditions.

In a series of experiments, samples of the fermentation products were reduced at a mercury pool electrode in a histidine buffer (composition as for operating system 3 but with no additive). A three-electrode arrangement was employed (carbon auxiliary electrode; SCE reference electrode), with a working potential of −1.5 V. The course of the reduction was monitored by ITP. Isotachopherograms for the separation of the anionic constituents present in the crude citric acid before and after electrochemical pre-treatment are given in Fig. 1. Fig. 2 illustrates the differentiating ability of the complex formation for this group of constituents.

An on-line electrochemical detector for ITP is still not available. Therefore, electrochemical sample pre-treatment or micropreparative fractionation followed by off-line electrochemical evaluation are the only alternatives.

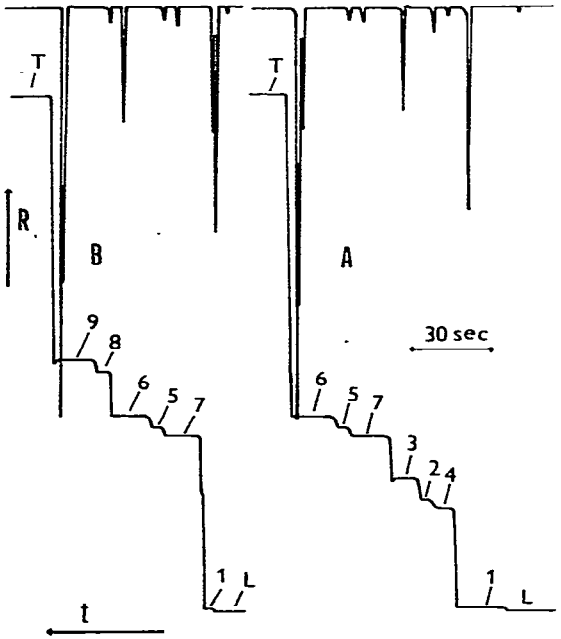

Fig. 1. Isotachopherograms for the separation of anions present in the crude citric acid before (A) and after (B) the electroreduction. Operating system 1. Driving current 45 μA. Acids: 1 = sulphuric; 2 = α-ketoglutaric; 3 = *trans*-aconitic; 4 = *cis*-aconitic; 5 = malic; 6 = isocitric; 7 = citric; 8 = α-hydroxyglutaric; 9 = 1,2,3-propanetricarboxylic. L = Chloride; T = acetate. t = Increasing time; R = increasing resistance.

Quantitative analysis

We are mainly interested in the amounts of citric and isocitric acids present in the analysed materials. As no other analytical method giving satisfactory quantitative results was available, only ITP determinations were carried out.

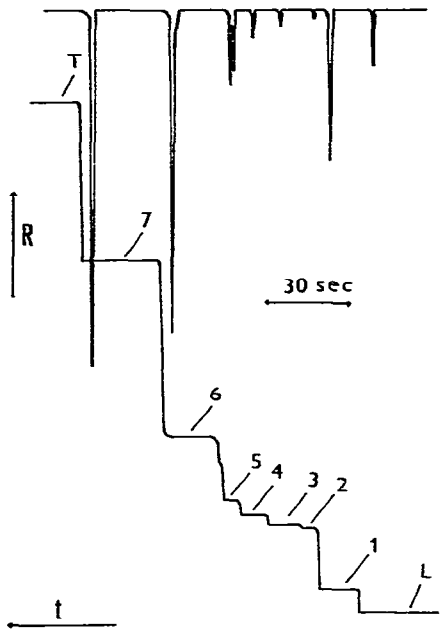

Fig. 2. Isotachopherograms for the separation of anions present in the crude citric acid, according to their complex formation with Ca^{2+}. Operating system 5. Driving current 45 μA. Symbols as in Fig. 1, except T = MES.

An ITP determination can be biased by a systematic analytical error:

when the constituent to be determined migrates in a zone containing another co-migrating constituent(s) in the total amount higher than the reproducibility of quantitation attained by ITP;

when a part of the material to be determined is lost, *e.g.*, due to inappropriateness of the chosen operating system, or when quantitation is made before the zone of the constituent is fully resolved.

In order to detect the appearance of such errors, quantitation was carried out in two operating systems which gave full resolutions of the sample constituents.

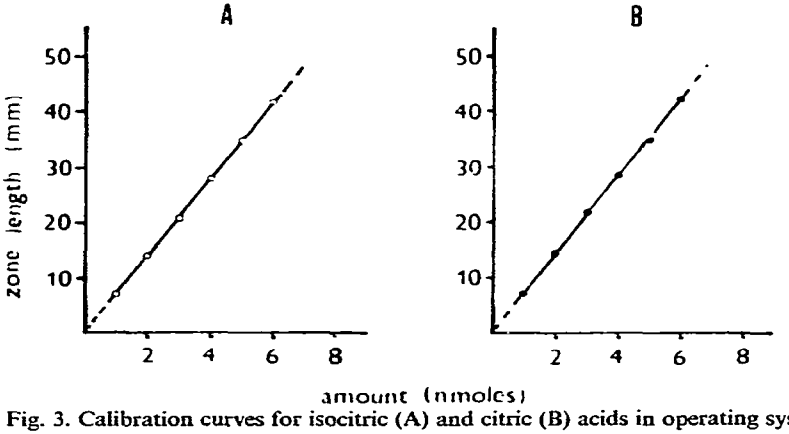

Fig. 3. Calibration curves for isocitric (A) and citric (B) acids in operating system 5.

Calibration curves for citric and isocitric acids in both systems were obtained for a model mixture similar in composition to actual samples, and that for operating system 5 is given in Fig. 3. The results of quantitative analyses for two arbitrarily chosen samples are summarized in Table II. The lower and upper levels for the 95% confidence interval represent 1–2% of the amount determined. Better reproducibilities (× 2–5) of the determinations of both constituents were obtained when the samples were dosed with the aid of a valve[11] instead of a microsyringe.

TABLE II

QUANTITATIVE ANALYSIS OF CITRIC AND ISOCITRIC ACIDS IN THE CRUDE CITRIC ACID

Means (mg/l) were calculated from four parallel determinations and the reproducibilities are expressed as the relative width of the reliability interval for the 95% confidence level.

Acid	*Systems*	
	1	*5*
Sample No. 1		
Citric	40.01 ± 0.40	40.40 ± 0.76
Isocitric	36.29 ± 0.75	36.20 ± 0.64
Sample No. 2		
Citric	42.46 ± 0.85	42.08 ± 0.30
Isocitric	21.52 ± 0.21	21.68 ± 0.22

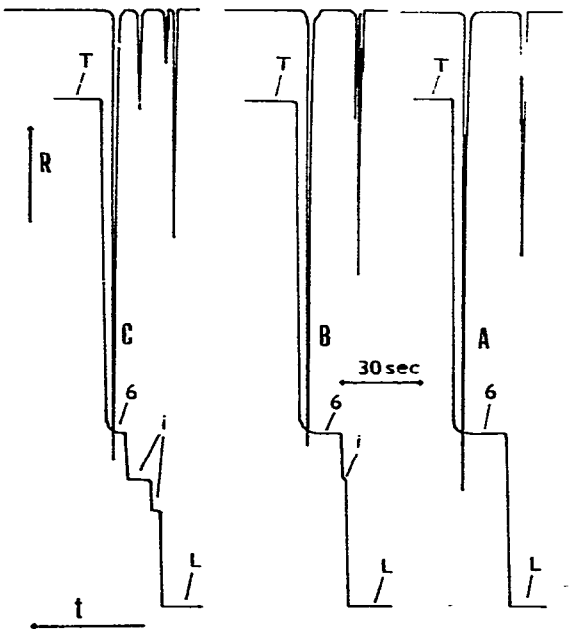

Fig. 4. Anionic ITP profiles of isocitric acid from different commercial sources in operating system 5. Driving current 45 μA. i = Impurities; A, B, C = different products. Other symbols as in Fig. 2.

The purity of isocitric acid from different commercial sources and in different purity grades varied unexpectedly widely, contents of 30–98% being typical. Of the available preparations that having the highest relative content of isocitric acid (trisodium salt of DL-isocitrate, A grade; Calbiochem-Behring) was used as a reference substance for the quantitative evaluation. The isotachopherograms in Fig. 4 clearly illustrate the different degrees of purity of some commercial preparatives of isocitric acid supplied as the sodium salts.

CONCLUSIONS

Analyses of more than 50 samples taken from the fermentation broth as well as of the crude product showed that ITP is an excellent technique for routine analysis of the production of citric acid from *n*-alkanes. Its main advantages are reliable qualitative and quantitative data, speed of analysis, simple instrumentation and low running cost. An instrument provided with a six-way sampling valve[11] and a capillary tube (8 cm × 0.3 mm I.D.) enabled the determinations of citric and isocitric acids in about 5 min with relative reproducibilities of 1% in operating system 5.

Identification of the constituents present in a mixture of unknown composition can be derived from an on-line detector whose response is related to the effective mobilities of the constituents (*e.g.*, conductimetric, potential gradient, thermometric). To achieve an acceptable reliability of the identification, the group of leading electrolytes should be chosen carefully. Differentiation of the constituents according to their p*K* values, ionic mobilities, stability constants and charges provided an unambiguous identification in this work.

The electrochemical sample pre-treatment monitored by ITP used in this work gives valuable information for qualitative analysis of electrochemically active components in samples of unknown composition.

Quantitation was performed in two operating systems which gave full resolutions of the constituents in order to detect any systematic errors.

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