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ISOTACHOPHORETIC DETERMINATION OF LYSINE IN FERMENTATION BROTHS

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SUMMARY

The use of capillary isotachophoresis for the determination of lysine present in fermentation broth and also in the crude product was investigated. Comparative analyses performed by isotachophoresis and a referee manometric method showed a high accuracy of isotachophoretic determinations. The coefficient of variation for the isotachophoretic determinations of lysine in the samples of interest was always well below 1% when the sample was injected with the aid of sampling valve and the time of the analysis was 4 min or less.

INTRODUCTION

L-Lysine is an important feed component and increasing demands for this essential amino acid have led to the development of large-scale fermentation production. Rapid, accurate and very reproducible determination of lysine is necessary for optimal process control and for the analysis of the crude product.

There are several methods suitable for the determination of lysine, including spectrometric and fluorimetric methods based on different chemical reactions[1,2]. High analytical selectivity and excellent accuracy and reproducibility are achieved using a manometric method in which enzymatically liberated CO_2 is measured[3–5]. However, the routine use of this method is not very convenient because the analysis time is long (25–30 min) and it is a laborious procedure.

Enzyme-selective electrodes, *e.g.*, as described by Macholán[6], have some advantages in this respect as they are rapid and simple to use. However, their reproducibility of quantitation (2–4% relative standard deviation) is low for precise process control purposes and is hardly acceptable for the analysis of the crude product.

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In the analysis of lysine and other amino acids present in complex physiological or reaction mixtures, separation methods are usually used, mainly with amino acid analysers employing ion-exchange column packing materials with post-column detection using derivatization with ninhydrin or with a fluorogenic reagent[7–9]. Reversed-phase high-performance liquid chromatography (RP-HPLC) offers at least similar analytical possibilities to highly specialized amino acid analyzers[10–12]. Gas–liquid chromatography (GLC) can also be used for the analysis of amino acids after their derivatization using one of several procedures[7,9]. The use of capillary isotachophoresis (ITP) for the analysis of amino acids was reviewed by Holloway and Pingoud[13] and Everaerts *et al.*[14].

Our aim was to develop a method suitable for the accurate, reproducible, simple and rapid determination of lysine present in fermentation broth and applicable also to the analysis of the crude product. The high reproducibility requirement (coefficient of variation for parallel determinations less than 1%) substantially reduced our choice, as methods characterized by high analytical selectiivity, *e.g.*, ion-exchange chromatography with ninhydrin derivatization[7], RP-HPLC using on-column *o*-phthalaldehyde derivatization[12] and other pre- and post-column derivatization reactions[8,9] rarely provide reproducibility of quantitative analysis characterized by coefficients of variation better than 2%. As it is mainly the reproducibility of the derivatization reactions that is responsible for these random errors, direct spectrometric or fluorimetric determinations cannot be expected to provide better results. Moreover, the time of analysis and/or expensive instrumentation also make some of the above methods less attractive.

For these reasons we investigated the use of ITP and in this paper we discuss its possibilities for the determination of lysine in fermentation samples.

EXPERIMENTAL

Instrumentation

ITP analyses were carried out in an instrument similar to that developed by Everaerts *et al.*[14]. This instrument was provided with a capillary tube of 0.30 mm I.D. made of fluorinated ethylene–propylene (FEP) copolymer. Conductivity detection was used for recording the isotachopherograms[15]. The samples were injected with the aid of an injection system similar to that described by Vacík and Everaerts[16].

Experiments in which both cationic and anionic profiles of the samples were of interest were carried out in a laboratory-constructed instrument with the column-coupling configuration of the separation unit[17].

Chemicals

Doubly distilled water circulating continuously through a column packed with a mixed bed ion-exchange resin was used for the preparation of the leading and terminating electrolyte solutions.

Sodium acetate, tetrabutylammonium perchlorate and acetic acid were obtained from Lachema (Brno, Czechoslovakia), morpholinoethanesulphonic acid from Sigma (St. Louis, MO, U.S.A.), β-alanine from Loba-Chemie (Vienna, Austria) and histidine from Reanal (Budapest, Hungary). The chemicals were purified by recommended methods[18].

Lysine (free base) and its hydrochloride were obtained from several manufacturers and all preparations were used without further purification.

RESULTS AND DISCUSSION

The material taken from the fermentation broth and also the final product contained ionic constituents that were not exactly specified. As some of the cationic constituents could interfere in the determination of lysine, our introductory experiments were focused on the determination of cationic ITP profiles within the pH range 5.0–9.5. The profiles were obtained using available operational systems[19]. These experiments showed that both sample types are cationically simple and the results of quantitative analyses performed in this pH range agreed well. A typical isotachophorogram obtained is shown in Fig. 1.

On the other hand, both the fermentation broth and the crude lysine were anionically complex, as illustrated by the isotachopherograms in Fig. 2. These results show clearly that the use of ITP for the analysis of lysine (which migrates as a cation) present in such an anionic matrix is very advantageous as possible anionic interferences are eliminated by the separation process.

A simple cationic profile provided a high degree of freedom in the choice of the operational systems. Of the possible alternatives we preferred those which could be prepared in high purity from readily available chemicals (for the compositions of the operational systems, see Table I). Ethylenediaminetetraacetic acid (EDTA) was added to the leading electrolytes to decrease the effective mobilities of Ca^{2+} and Mg^{2+} and thus minimize the overall requirements for the load capacity[20].

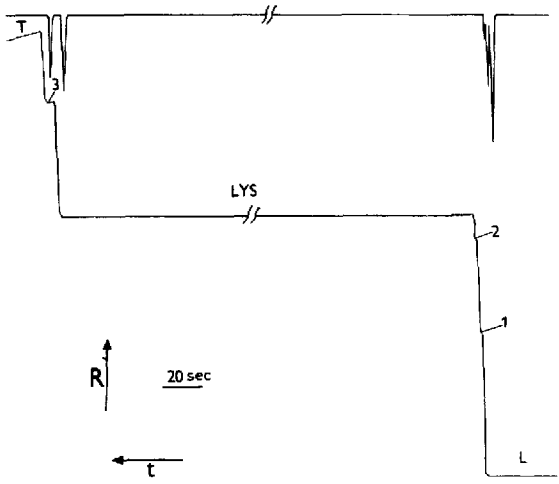

Fig. 1. Isotachopherogram from the cationic profile experiments. Only the traces from the analytical column are given. In this experiment only the constituents more and less mobile than lysine were introduced into the analytical column with a smaller amount of lysine. The separation was performed with operational system No. 1 (Table I). The driving current in the pre-separation column was 200 μA and that in the analytical column was 45 μA. L,T = leading and terminating electrolytes, respectively. R = increasing resistance; t = increasing time. 1–3 = unidentified cationic constituents present in the lysine sample taken from the fermentation process.

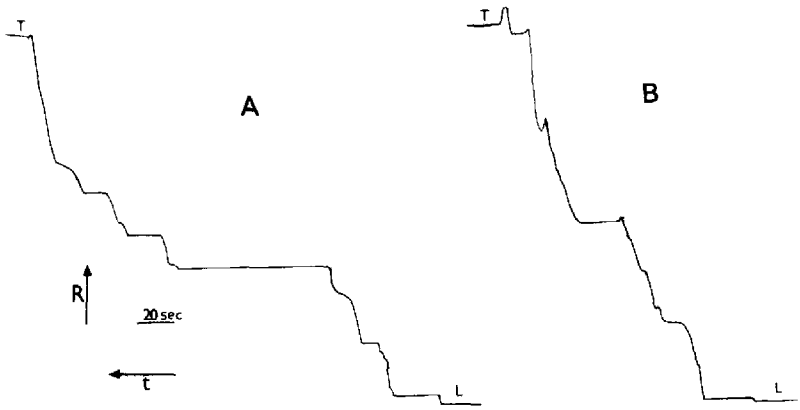

Fig. 2. Isotachopherograms from the anionic experiments. A,B = separation with operational systems Nos. 4 and 3, respectively. Only the isotachopherograms from the analytical column are given. The driving currents were 200 and 40 μA in the pre-separation and analytical columns, respectively. The amount of the sample was the same as in cationic profile experiments (Fig. 1). The constituents migrating within the leading–terminating mobility intervals were not identified. For the meaning of the other symbols, see Fig. 1.

TABLE I

OPERATIONAL SYSTEMS

Ac^- = acetate; EDTA = ethylenediaminetetraacetic acid; MES = morpholinoethanesulphonic acid; HEC = hydroxyethylcellulose; TBA = tetrabutylammonium perchlorate; BALA = β-alanine; HIS = histidine.

Parameter	*Cationic analysis*	
	System 1	*System 2*
Solvent	Water	Water
Leading cation	Na^+	Na^+
Concentration (m*M*)	10	10
Counter ion	Ac^-	MES^-
Co-counter ion	EDTA	EDTA
Concentration (m*M*)	1	1
Additive to the leading electrolyte	HEC (0.1%)	HEC (0.1%)
pH of leading electrolyte	5.04	6.16
Terminating cation	TBA^+	TBA^+
Concentration (m*M*)	5	5
	Anionic analysis	
	System 3	*System 4*
Solvent	Water	Water
Leading anion	Cl^-	Cl^-
Concentration (m*M*)	10	10
Counter ion	BALA	HIS
Additive to the leading electrolyte	HEC (0.1%)	HEC (0.1%)
pH of the leading electrolyte	3.05	6.02
Terminating anion	Ac^-	MES^-
Concentration (m*M*)	5	5

TABLE II

COMPARISON OF DIFFERENT SAMPLE INTRODUCTION TECHNIQUES

Microsyringe			*Valve*		
Zone length (sec)*	*S.D. (sec)*	*C.V. (%)*	*Zone length* (sec)*	*S.D. (sec)*	*C.V. (%)*
14.69	0.36	2.4	13.72	0.09	0.66
29.44	0.72	2.4	29.38	0.18	0.43
41.94	0.72	1.7	41.91	0.18	0.43
57.69	0.90	1.5	57.19	0.09	0.16

* Zone lengths were measured with a stop-watch as the time between the peaks of the differential conductimetric signal that defined the zone of lysine; the means of six parallel runs are given.

Our main aim was to develop a procedure suitable for the routine analysis of lysine and applicable for both process control and analysis of the crude product. With respect to a simple cationic profile of the samples, a single column arrangement of the separation unit was preferred and performance characteristics such as accuracy, reproducibility and time of analysis were investigated to establish the optimal configuration of such a separation unit for routine use.

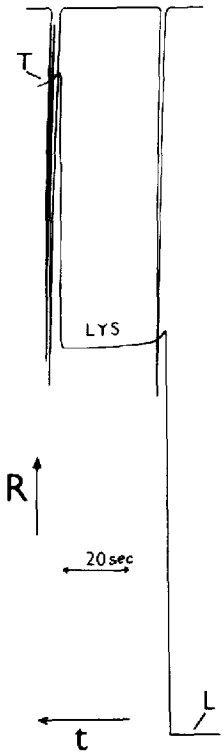

Fig. 3. Typical isotachopherogram from the analysis of lysine present in fermentation broth. A single column separation unit was used with operational system No. 2 (Table I). The driving current was 45 μA. For the meaning of the symbols, see Fig. 1.

TABLE III

ANALYSIS OF DIFFERENT LYSINE PREPARATIONS BY ITP

Reference material No.	*Content of lysine in the crude product** (% w/w)	*Content of lysine stated by manufacturer (% w/w)*	*Purity by ITP*** (% w/w)
1	48.33 ± 0.16	80	68.1
2	38.65 ± 0.13	–	85.2
3	36.58 ± 0.12	98	90.0
4	32.91 ± 0.11	99.2	99.2

* The content when a particular reference material is taken as 100% pure.

** Actual content of lysine as determined with respect to reference material No. 4 (lysine hydrochloride).

In a series of experiments, different amounts of the crude product were analyzed in a 25-cm long capillary tube to determine the minimal load capacity of the separation system required for the determination of lysine with a reproducibility less than 1% (coefficient of variation). The sample solutions were introduced into the analyser with the aid of the sampling valve or using a 10-μl microsyringe. In this series of experiments, therefore, both injection techniques could be compared at the same time. The resulsts of this comparison (see Table II) show that the use of valve enabled the desired reproducibility to be achieved for shorter zones. Consequently, a short capillary tube allowing an analysis time of 3–4 min (for a fully resolved zone of lysine migrating for *ca.* 30 sec through the detection cell) could be proposed for routine use. An example of a typical isotachopherogram from the determination of lysine is given in Fig. 3.

As the aim of this work was also to evaluate the accuracy of the ITP procedure with respect to a referee manometric method[3–5], a proper choice of the reference

TABLE IV

COMPARISON OF ITP DETERMINATION OF LYSINE WITH A REFEREE MANOMETRIC METHOD

Lysine (%, w/w)			
	*ITP, Operational system No. 1**	*ITP, operational system No. 2**	*Manometry*
	33.25	33.34	33.52
	33.53	33.44	33.46
	33.60	33.26	33.49
	33.17	33.33	33.17
	33.15	33.12	33.20
	33.54	33.13	33.07
Mean (%)	33.27	33.29	33.32
S.D. (%)	0.18	0.13	0.18
C.V. (%)	0.54	0.39	0.54

* Means of two parallel runs on the same stock solution; six stock solutions were prepared by weighing both lysine and water (added to the proper dilution for the analysis).

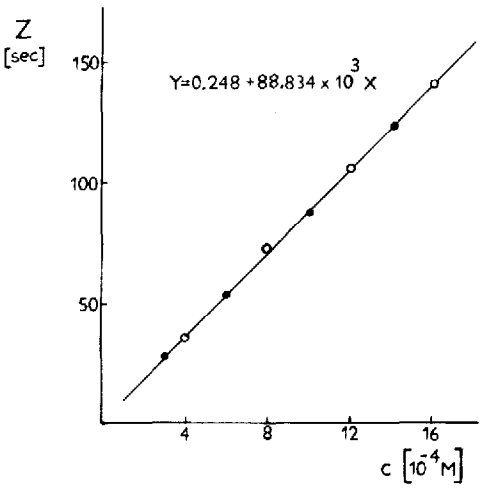

Fig. 4. Calibration graph for the determination of lysine with operational system No. 2 (Table I). ○, Original points of the calibration graph; ●, calibration points from the evaluation of the validity of the calibration graph after 1 month.

material was essential and different commercial lysine preparations were tested for this purpose. Lower contents of lysine than claimed by the manufacturers were found in some of them and that with the highest actual content was accepted as a reference substance (see Table III). The high hygroscopicity of free lysine base and its ability to adsorb carbon dioxide may explain the lower contents found. For these reasons the use of lysine hydrochloride was preferred in our work.

For the evaluation of the accuracy of the ITP method one sample of the crude product was repeatedly analysed in our laboratories. A lysine hydrochloride preparation containing 99.2% of the substance was used as the reference material. ITP determinations were carried out in both cationic operational systems (Table I) and in each of them the same crude lysine sample was analysed six times. Manometric determinations were carried out as described in the literature[3–5] and the same sample was analysed six times. The results of these comparative analyses are summarized in Table IV. These results showed excellent agreement for both ITP systems and for ITP and manometry.

The content of lysine present in samples taken from the fermentation broth and in the crude products was evaluated from a calibration graph. As the same instrument was used during this work and the other analytical conditions were kept constant, the validity of the calibration graph was checked each day only for two points (lower and upper contents). Once monthly a complete set of measurements was carried out to evaluate the validity of the calibration graph. In all instances excellent agreement of the results was achieved, especially when weighing was used instead of volumetric procedures for the preparation of the calibration solutions (see Fig. 4).

CONCLUSIONS

The determination of lysine present in samples from fermentation production can be carried out by ITP with several advantages. The method developed in this work fulfills all the requirements necessary for a routine procedure, *i.e.*, high accuracy, high reproducibility, simple instrumentation and almost no sample pre-treatment (only filtration). With respect to the small number of cationic constituents accompanying lysine in the samples, a low load capacity is required. Consequently, the time of analysis (less than 4 min) is comparable to that with an enzyme-selective electrode[6].

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