Journal of Chromatography, 638 (1993) 241–246
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 861

Capillary isotachophoresis of organic acids produced by selected microorganisms during lactic acid fermentation

J. Karovičová, J. Polonský*, M. Drdák, P. Šimko and V. Vollek

Faculty of Chemical Technology, Slovak Technical University, Radlinského 9, 812 37 Bratislava (Slovak Republic)

ABSTRACT

Organic acids present in samples of vegetables [green peas, carrots, celery, paprika (capsicum) and onions] after lactic acid fermentation by selected microorganisms were determined by capillary isotachophoresis. Lactic, acetic, phosphoric, citric, propionic and butyric acid and potassium sorbate were determined.

INTRODUCTION

Preservation by lactic fermentation uses the ability of microorganisms to produce substances that prolong the natural storage time of foods [1]. The objectives for using lactic acid bacteria are to make the food durable, to improve its taste and to maintain the nutritive, physiological and hygienic value of the fermentation products. Lactic fermentation is a complex microbiological process that is influenced by using factors, such as the concentration of salt, temperature, microorganisms and air exclusion [2].

The by-products of lactic fermentation consist mainly of acetic acid with small amounts of formic, propionic, valeric, succinic and caproic acid. They contribute to finishing and rounding of the taste. Butyric acid, produced by bacteria of lactic fermentation, has an adverse effect owing to its particular smell and is therefore undesirable [3,4].

There is commercial interest in the production of vegetable juices fermented by lactic fermentation. Spontaneous fermentation or inoculation of selected and tested cultures of microorganisms is used for the production of the juices. Cultures of microorganisms are required that deepen the aroma and make possible a rapid decrease in the pH of the juices. Lactic fermentation is suitable especially for processing vegetables, mainly cabbage and cucumbers, but also sugar beet, carrots, cauliflower, kohlrabi, paprika (capsicum), onions, asparagus and vegetable mixtures.

Many workers are engaged in research on new products fermented by lactic fermentation, either conserved vegetables or vegetables or fruit juices fermented by lactic fermentation [5–7]. Yu [8] effected the lactic fermentation of soya milk by the strains *Lactobacillus casei* and *Kluyveromyces fragilis*. Manan [9] accomplished laction fermentation of potato slices in salty brine. Ogbadu and Okagbue [10] were engaged in lactic fermentation of soy beans with a starting culture of *Lactobacillus plantarum*. Valdez *et al.* [11] achieved the lactic fermentation of green peas, sweet black pepper and cabbage. Oyewole and Odunfa [12] divided and characterized the cultures of lactic fermentation.

Methods for the identification and determination of organic acids in foods and their advantages, including capillary isotachophoresis, were published by Karovičová and co-workers [13,14]. The aim of this study was to follow the production of organic acids, especially lactic acid and also acetic acid, during lactic fermentation of

* Corresponding author.

0021-9673/93/$06.00

vegetables by different microorganisms. On the basis of results obtained for the production of lactic acid, suitable microorganisms can be selected for use in the production of fermented vegetables that can be employed for the production of vegetables for preparing vegetable drinks or salads.

Capillary isotachophoresis, owing to its advantages (simple sample preparation, short time of analysis), is comparable to other chromatographic methods and is suitable for the determination of organic acids.

EXPERIMENTAL

Fermentation

Fermentation experiments were carried out with different kinds of vegetables: green peas, carrots, celery, paprika (capsicum) and onions. The experiments were performed in fermentation flasks in such a way that after the inoculation of a microorganism anaerobic conditions were maintained. The fermentation lasted 7–10 days at 23–26°C.

For the inoculation of fermented vegetables in the fermentation flasks, the following lyophilized microorganisms were used: *Lactobacillus* sp. "S", $3.04 \cdot 10^8$ microorganisms (MO)/ml; *Pediococcus acidilactici*, $3.52 \cdot 10^7$ MO/ml; *Pediococcus* sp. "CSL", $2.05 \cdot 10^7$ MO/ml; *Lactobacillus fermentum*, $7.08 \cdot 10^7$ MO/ml; *Lactobacillus* sp. "U", $2.60 \cdot 10^7$ MO/ml; *Lactobacillus* spp., $5.40 \cdot 10^8$ MO/ml; *Lactobacillus bucheri*, $1.32 \cdot 10^8$ MO/ml; and *Lactobacillus brevi*, $2.40 \cdot 10^8$ MO/ml.

For the experiments the cultivation base according to Rogos was used [19]. The cultivation lasted 3 days at 37°C.

Fermentation of green peas. Frozen green peas were used. Brine of the required concentrations of salt and reducing sugars was prepared after thawing and after the basic analyses (determination of reducing sugars, titriatable acids, pH). The concentration of reducing sugars for samples A1–3 was 2.40% and the adjusted concentration of reducing sugars for samples A4–7 was 3.57%. The brine in samples A1–7 was 1.5% NaCl and the ratio of mixing the brine and green peas was 5:4. The fermentation lasted 7 days at 23°–26°C.

Fermentation of carrots. Stored, cleaned and grated carrots with brine of 1.5% NaCl were used. For samples B1–8, 0.1% $CaCl_2$ and 0.1% potassium sorbate were added; sample B9 was without potassium sorbate. Better anaerobic conditions were created by nitrogen bubbling. The ratio of carrots to brine was 5:9. The fermentation lasted 10 days.

Fermentation of celery. Stored celery was cut into cubes and immersed in brine (celery to brine ratio = 3:4) before fermentation. For samples C1–5 0.1% potassium sorbate was added and for samples C3–5 nitrogen bubbling was also applied. A higher concentration of phytoncide substance (0.01%) was applied to the C6–8 and a lower concentration (0.005%) to samples C9 and 10. The brine used for all samples C1–10 was 1.5% NaCl.

Fermentation of paprika (capsicum). Frozen paprika was cut into strips and immersed in brine (paprika to brine ratio = 4:5) before fermentation. The brine contained 1.5% NaCl and 0.1% $CaCl_2$ for samples D1–8, and was also 0.1% potassium sorbate for samples D3 and 4. A higher concentration of phytoncide substance (0.01%) was applied to samples D5 and 6 and a lower concentration (0.005%) to samples D7 and 8. The fermentation lasted 10 days.

Fermentation of onions. Onions (E) were cleaned and cut into round pieces and immersed in brine (onion to brine ratio = 2:3) before fermentation. The brine was 1.5% NaCl. The fermentation lasted 7 days.

Isotachophoresis

Fermented samples were homogenized and filtered before isotachophoretic analyses. Measurements were made on a CS ZKI 01 isotachophoretic analyser (Spišská Nová Ves, Slovak Republic) equipped with a conductivity detector and a TZ 4200 double line recorder.

The samples were analysed at a driving current of 200 μA in the preseparation column and 50 μA in the analytical column. For identification and determination the electrolytic system applied had the following composition: concentration of leading electrolyte, 0.01 *M* HCl; counter ion, ε-aminocaproic acid; pH, 4.5; additive, methylhydroxyethylcellulose (0.1%); terminat-

TABLE I

CHARACTERISTIC CONSTANTS OF THE STANDARDS OF ORGANIC ACIDS AND THE PARAMETERS OF THE CALIBRATION LINES ($y = a + bx$)

R.s.h. = Relative step height = $(h_i - h_L)/(h_T - h_L)$, where h_i is the line of the ith ion, h_L is the line of the leading electrolyte and h_T is the line of the terminating electrolyte; r = correlation coefficient; a = intercept on ordinate (mm); b = slope (mm/mmol).

Organic acid	R.s.h.	a	b	r
Phosphoric acid	0.299	2.4163	9.5581	0.9971
Lactic acid	0.310	−1.6584	17.5145	0.9968
Acetic acid	0.327	1.7064	11.5406	0.9916
Citric acid	0.227	−0.0988	19.8256	0.9973
Propionic acid	0.600	0.7776	17.1076	0.9989
Butyric acid	0.777	0.9442	16.7729	0.9994

ing electrolyte, $5 \cdot 10^{-3}$ M caproic acid–$5 \cdot 10^{-3}$ M histidine (pH 4–5).

Samples diluted 1:25 with water were injected into the column using the four-way valve of the instrument. The duration of the analysis was 20–30 min. Quantitative analysis was performed by calibration. Based on the presumed presence of organic acids, standard solutions of lactic, acetic, citric, phosphoric, propionic and butyric acid and potassium sorbate of concentration 0.01 M were prepared. Values of the characteristic constants (relative step heights) of the standards of organic acids and the parameters of the calibration lines are given in the Table I.

RESULTS AND DISCUSSION

The study was aimed at the investigation of the suitability of the various microorganisms as starting cultures during the lactic fermentation of vegetables. The selection of the microorganisms was based mainly on the production of lactic acid and also on the sensory evaluation of the fermented vegetables. We wanted to retain the hardness of the vegetables and to gain a typical sour taste and adequate aroma. The designations of the analysed samples, the microorganisms used, and the average contents of the determined organic acids calculated from three measurements are given in Tables II–VI. The samples were always taken after the fermentation had finished.

The results of isotachophoretic analysis of the samples of green peas are presented in Table II. Only lactic and acetic acid were present in the samples of green peas. The measured amount of lactic acid ranged from 6.75 to 14.56 g l^{-1}. The best producer of lactic acid was the microorganism *Pediococcus* sp. "CSL". Its production was the highest at the different concentrations of reducing sugars. This microorganism also showed a very suitable production of acetic acid, which gave a good taste and aroma to the product.

Recently there has been a demand to lower the amounts of chemical preservatives added to food products. With regard to this problem, our task was to establish and use the preservation effects of phytoncides at two different concentrations. Phytoncides inhibit the growth of many microorganisms. They may have different chemical compositions, taste and aroma and many are characteristic, strong and pervasive. They are components of some spices and herbs. Phytoncides are the subject of research as additional preservation agents. We obtained products with a pleasant sour taste, excellent appearance and the same hardness after fermentation for 1 week with application of phytoncides.

Table III gives the organic acids in the samples of fermented carrots after 10 days of fermentation. We tested a large number of microorganisms during the fermentation of carrots. We identified lactic, acetic, phosphoric, citric, propi-

TABLE II

ORGANIC ACIDS IN GREEN PEA SAMPLES

Sample	Microorganism	Contents of organic acids (g l^{-1})	
		Lactic	Acetic
A1	*Pediococcus* sp. "CSL"	9.67	2.83
A2	*Lactobacillus* sp. "S"	7.24	1.69
A3	*Pediococcus acidilactici*	6.75	2.36
A4	*Lactobacillus* sp. "S"	11.64	5.40
A5	*Pediococcus acidilactici*	7.77	6.85
A6	*Pediococcus* sp. "CSL"	14.56	4.90
A7	*Lactobacillus* sp. "U"	14.52	5.64

TABLE III

ORGANIC ACIDS IN CARROT SAMPLES

Sample	Microorganism	Contents of organic acids (g l^{-1})					
		Lactic	Acetic	Phosphoric	Citric	Propionic	Butyric
B1	*Lactobacillus buchneri*	8.80	4.59	0.19	1.47	–	–
B2	*Pediococcus* sp. "CSL"	13.21	4.85	0.15	–	–	1.31
B3	*Lactobacillus* sp. "U"	8.04	3.28	–	–	0.43	–
B4	*Lactobacillus fermentum*	9.69	4.28	–	–	–	–
B5	*Lactobacillus* spp.	7.53	2.56	–	–	0.24	0.27
B6	*Pediococcus acidilactici*	7.01	3.32	–	–	0.36	0.45
B7	*Lactobacillus* brevi	4.89	4.60	–	0.18	0.79	–
B8	*Lactobacillus* sp. "S"	9.36	4.19	1.47	0.42	0.62	–
B9	*Lactobacillus* sp. "S"	8.39	2.40	–	–	0.22	–

onic and butyric acid in the individual fermentation samples. Under the same conditions, *Lactobacillus fermentum*, *Lactobacillus* sp. "S" and *Pediococcus* sp. "CSL" produced the most lactic acid, but butyric acid was also produced, which is not desirable for the sensory aspect of the product. The measured amounts of lactic acid ranged from 4.89 to 13.21 g l^{-1}. Fig. 1 shows the analysis of a carrot sample.

Table IV lists the organic acids present in the samples of fermented celery. The samples were taken after 10 days of fermentation. Lactic, acetic, phosphoric, citric and propionic acid and potassium sorbate were identified. A concentration of 0.98 g l^{-1} of potassium sorbate was determined in sample C2. Most lactic acid was determined in sample C2 with the microorganism *Lactobacillus* sp. "S", and similar results were obtained with samples C3–5. Nitrogen bubbling was also used in the fermentation of celery. We found that the product without nitrogen bubbling had a typical sour taste and aroma. We also tried omitting the use of preservatives with this product. We tested two different concentrations of phytonicide substance. With regard to the sensory aspect, both tested concentrations of phytoncide substance were convenient for this product.

Lactic, acetic and propionic acid were determined by isotachophoretic analysis of the samples of paprika (Capsicum) (Table V). A 0.98 g l^{-1} concentration of potassium sorbate was determined in samples D3 and 4. The highest content of lactic acid in samples D1–4 was in sample D2 when *Lactobacillus* sp. "S" was used, but at the same time there was also a fairly high content of acetic acid (up to 5.08 g l^{-1}).

In the literature there are numerous contradictory discussions about the effect of acetic acid

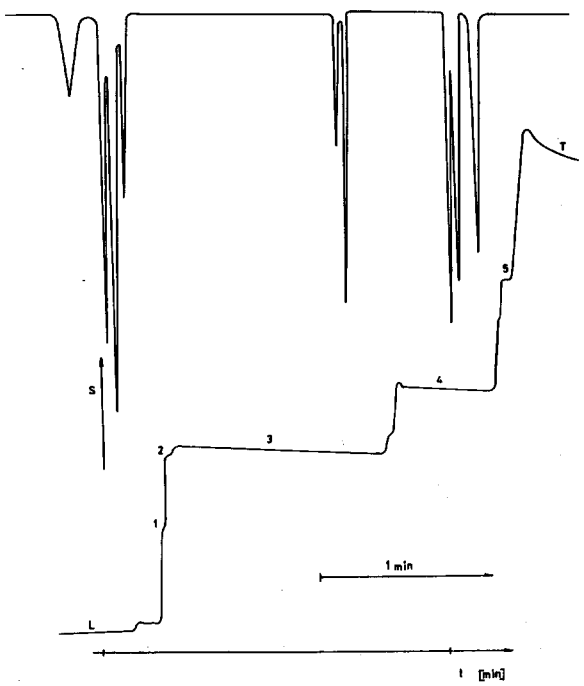

Fig. 1. Isotachopherogram of organic acids in the carrot B8 sample. *S* = Response of conductivity detector; L = leading electrolyte; T = terminating electrolyte. 1 = Citric acid; 2 = phosphoric acid; 3 = lactic acid; 4 = acetic acid; 5 = propionic acid.

TABLE IV

ORGANIC ACIDS IN CELERY SAMPLES

Sample	Microorganism	Contents or organic acids (g l^{-1})				
		Lactic	Acetic	Phosphoric	Citric	Propionic
C1	*Lactobacillus* sp. "U"	9.23	4.84	0.65	0.99	0.34
C2	*Lactobacillus* sp. "S"	11.42	2.65	0.63	1.38	0.51
C3	*Lactobacillus* sp. "U"	6.48	3.28	0.40	0.68	0.69
C4	*Lactobacillus* sp. "S"	8.47	3.34	0.67	0.69	0.51
C5	*Lactobacillus fermentum*	7.03	3.13	0.19	0.70	0.61
C6	*Lactobacillus* sp. "S"	13.39	7.77	0.41	0.95	–
C7	*Lactobacillus fermentum*	11.29	6.48	1.08	0.69	–
C8	*Lactobacillus* sp. "U"	9.38	6.47	–	0.69	–
C9	*Lactobacillus fermentum*	10.76	8.00	0.19	0.68	–
C10	*Lactobacillus* sp. "U"	8.80	7.46	1.10	0.69	–

TABLE V

ORGANIC ACIDS IN PAPRIKA (CAPSICUM)

Sample	Microorganism	Contents of organic acids (g l^{-1})			
		Lactic	Acetic	Citric	Propionic
D1	*Lactobacillus fermentum*	7.49	5.08	0.82	0.27
D2	*Lactobacillus* sp. "S"	7.61	4.86	0.69	0.27
D3	*Lactobacillus fermentum*	6.44	4.65	0.95	0.27
D4	*Lactobacillus* sp. "S"	6.60	4.55	0.69	0.27
D5	*Lactobacillus* sp. "S"	7.18	2.15	0.82	0.21
D6	*Lactobacillus fermentum*	7.34	3.24	0.69	0.22
D7	*Lactobacillus* sp. "S"	6.89	2.03	0.69	0.31
D8	*Lactobacillus fermentum*	4.09	1.34	0.69	0.31

concentration on the quality of fermented products. It is known that the absence of acetic acid leads to products which have a unilaterally sour taste and an atypical and flat aroma [15]. Similarly to the samples of celery, paprika was also fermented with addition of phytoncide substance. *Lactobacillus fermentum* (D6) was the best producer of lactic acid in samples D5–8. During the experiments, 0.1% $CaCl_2$ was always added to the brine to maintain the hardness of the product.

Lactic, acetic, phosphoric, citric and propionic acid were determined by isotachophoretic analysis of samples of onions. The average values are given in Table VI. The highest content of lactic acid was present in sample E1 (7.71 g l^{-1}) with the use of *Lactobacillus* sp. "S". After fermentation for 1 week onions had pleasant taste, became slightly softer and were suitable for salads or for the production of vegetable drinks. Butyric acid at 0.45 g l^{-1} was also determined in sample E2 with the use of *Lactobacillus fermentum*, which adversely influenced the sensory characteristics of the product.

The production of lactic acid during the cultivation time period indicated that the production of lactic acid depends on the type of microorganism used and not directly on time.

From the evaluation of the effect of NaCl we can say that an increased NaCl concentration does not affect the activity of microorganisms unfavourably. As some fermented products were already softened after 2 days of storage, we added 0.1% $CaCl_2$ to subsequent samples. Mi-

TABLE VI

ORGANIC ACIDS IN ONION SAMPLES

Sample	Microorganism	Contents of organic acids (g l^{-1})					
		Lactic	Acetic	Phosphoric	Citric	Propionic	Butyric
E1	*Lactobacillus* sp. "S"	7.71	2.40	–	–	–	–
E2	*Lactobacillus fermentum*	2.61	1.53	0.65	2.23	0.31	0.45
E3	*Lactobacillus* sp. "U"	6.32	1.34	–	2.23	0.31	–
E4	*Lactobacillus* spp.	6.81	1.34	0.30	1.20	0.27	–

croorganisms produce only a small amount of acids at a low concentration of sugar. If the total concentration of reducing sugars is so low that the pH drops to 4.1 after the fermentation, butyric acid may be produced, which worsens the sensory characteristics of the product. Hence it is necessary to ensure that the content of reducing sugars is about 4% in the whole product before fermentation.

For the determination of organic acids in individual fermented samples, the standard deviations s_x [16] were as follows: lactic acid, 0.06–0.09; acetic acid, 0.09–0.19; phosphoric acid, 0.01–0.15; citric acid, 0.01–0.07; and propionic acid, 0.01–0.02 g l^{-1}. The relative standard deviations s_r were 0.62–2.55%.

In conclusion, capillary isotachophoresis is a suitable method for the determination of organic acids in foods [17,18] and fermented samples, because it gives information about the production of all organic acids and on the use of selected microorganisms in a relatively short time.

Other methods for the determination of organic acids have various disadvantages when compared with isotachophoretic methods. They often require special processing of the samples (derivatization, matrix elimination), which increases the time of analysis and also decreases the accuracy of determination.

Controlled fermentation should satisfy the sensory demands of users and it should also enrich the products with substances that are essential for humans. In this case the fermentation process starts earlier and nutritively important substances are preserved to a greater extent. This is why it is necessary to monitor especially the content of lactic, acetic and other organic acids which are produced by fermentation and essential and total amino acids and vitamins of group B in products fermented by lactic fermentation.

REFERENCES

1 V. Kyzlink, *Základy Konzervace Potravin*, SNTL, Prague, 1980, p. 513.
2 M. Drdák, *Technológia Rastlinných Neudržných Potravín*, Alfa, Bratislava, 1989, p. 300.
3 R. Binnig, *Flüssiges Obst., Sonderheft*, 3a (1982) 204.
4 H.P. Fleming and R.F. McFeeters, *Food Technol.*, 1 (1981) 84.
5 K. Gierschner, *Flüssiges Obst.*, 58 (1991) 236.
6 H. Buckenhüskes, B. Schwartz and K. Gierschner, *Dtsch. Lebensm.-Rundsch.*, 83 (1987) 319.
7 L.M. Beltrán-Edeza and H. Hernandéz-Sánchez, *Lebensm.-Wiss. Technol.*, 22 (1989) 65.
8 J.H. Yu, *Korean J. Food Sci. Technol.*, 20 (1988) 518.
9 J.K. Manan, *J. Food Sci. Technol.*, 24 (1987) 139.
10 L. Ogbadu and R.N. Okagbue, *J. Appl. Bacteriol.*, 65 (1988) 353.
11 G.F. Valdez, G.S. Giori, M. Garro, F. Mozzi and G. Oliver, *Microbiol. Aliment. Natur.*, 8 (1990) 175.
12 O.B. Oyewole and S.A. Odunfa, *J. Appl. Bacteriol.*, 68 (1990) 145.
13 J. Karovičová, M. Drdák and J. Polonský, *J. Chromatogr.*, 509 (1990) 283.
14 J. Karovičová, J. Polonský and A. Príbela, *Nährung*, 34 (1990) 665.
15 H. Buckenhüskes, A. Gessler and K. Gierschner, *Ind. Obst- Gemüseverwert.*, 73 (1988) 454.
16 K. Eckschlager, I. Horsák and Z. Kodejš, *Vyhodnocování Analytických Výsledků a Metod*, SNTL, Prague, 1980, p. 224.
17 L. An and Z. Yan, *Shipin Kexue*, 112 (1989) 1.
18 Z. Stránský, P. Peč, M. Kudlová, M. Mergl and J. Žabková, *Živočišna Výroba*, 33 (1988) 211.
19 *Potravinářské rýrobky, Stanovení počtu baktérií rodu Lactobacillus*, ČSN 56 0094, 1988.