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Ion-paired RP-HPLC for determining fermentation end products of *Streptococcus mutans* grown under different conditions

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ABSTRACT: A rapid, specific and reliable ion-paired high performance liquid chromatographic assay of small organic acids in bacterial cultures was developed. The assay was conducted using a mixture of 6.0×10^{-3} mM tetrabutylammonium phosphate and 5 % v/v acetonitrile in water as the mobile phase. Calibration curves for acetic, lactic, and pyruvic acid were linear within the concentration ranges determined ($r^2 > 0.9990$). Selective removal of interfering compounds from bacterial cultures improved the reliability of the method, demonstrating that more than 90% of the tested acids were recovered. Replicate regression analyses of acid standard plots obtained on three different days gave correlation coefficients of $r^2 > 0.9995$. The assay was precise within a day and between days as indicated by an ANOVA test. According to the validation results and from the analysis of the cultured samples, the described method may be adequate for the routine determination of acid metabolites of lactic acid bacteria.

KEYWORDS: lactic acid, acetic acid, formic acid, pyruvic acid, small organic acid, ion-paired RP-HPLC, *Streptococcus mutans*, lactic acid bacteria

INTRODUCTION

Streptococcus mutans, one of lactic acid bacteria (LAB), has been the principal agent causing human dental caries. It is capable of metabolizing a wide variety of carbohydrates ranging from mono-, di-, and tri-saccharides to high molecular weight dextran, fructan, and starch[1]. Acidification of the local environment by the end products of carbohydrate metabolism inhibits many competing bacterial species, enabling *S. mutans* to maintain its niche, and incidentally causing demineralization of tooth enamel[2]. To complement the number of carbohydrates *S. mutans* can use, it devotes a large portion of its coding potential (~15%) to various transport mechanisms[3]. For instance, sucrose is taken up by three distinct systems with K_m values of 7.1×10^{-5}, 2.5×10^{-4}, and 3.3×10^{-3} mM for sucrose-specific phosphotransferase (PTS), the trehalose-PTS, and a 'third transport system', respectively[4].

Pyruvate is the central intermediate of glycolysis, which is converted into a variety of acid end products depending on growth environments. In the presence of excess glucose under anaerobic conditions, *S. mutans* utilizes lactate dehydrogenase to convert pyruvate into lactic acid along with the oxidation of NADH to NAD^+. When glucose is limited, however, the bacterium relies on the pyruvate formate-lyase (PFL) pathway, which has two branches leading either to the formation of formic acid and acetic acid or to the formation of ethanol[5]. The ethanol branch involves the oxidation of 2NADH to $2NAD^+$, while an extra-ATP is generated in the acetate branch. By the appropriate use of the acetate and ethanol branches of the PFL pathway, *S. mutans* can readily adjust NADH oxidation to the actual need. Without further anabolic diversion of the final products, the amount of formic acid produced by glycolysis is equivalent to the total amount of acetic acid and ethanol[6].

An important characteristic which distinguishes *S. mutans* from other oral streptococci is its ability to ferment sugar alcohols such as sorbitol, and mannitol. One mole of them is degraded to 2 moles of pyruvate with a concomitant generation of 3 moles of NADH[7]. For smooth operation of glycolysis, NADH has to be oxidized to NAD^+. Since lactate dehydrogenase can

oxidize only 2 moles of NADH, *S. mutans*-PFL may play an important role in maintaining the intracellular balance of NADH and NAD^+ in the anaerobic metabolism of sugar alcohol[8]. Due to being extremely sensitive to oxygen, PFL can be inactivated by the penetrated O_2 of a thin dental plaque. Therefore, NADH has to be oxidized by another pathway[7]. Since the redox-potential falls during plaque development, deep layers of dental plaque are expected to be highly anaerobic. When a little sugar is supplied, especially between meals or during sleeping hours, *S. mutans* is able to obtain sufficient energy through the PFL pathway under anaerobic conditions of dental plaques. These could be reasons why *S. mutans* is highly cariogenic. Recently, much research has been devoted to studying biochemical and physiological adaptations that allow *S. mutans* to produce acids and to survive at low pH in the oral cavity.

Quantitative determination of the fermentation end products can be an additional support to estimated glycolytic pathways of microorganisms. Lactic acid and acetic acid were analysed by gas-liquid chromatography (GLC) following ether extraction[9]. Formic acid was quantified separately after conversion to dimethylformamide[10]. These methods had potential disadvantages; a lot of at least two sample preparation steps were needed, much time was required for the single run (~40 min), and interpretation of the chromatograms was sometimes difficult because of interference from a large solvent peak. An enzymatic determination of lactic acid has also been developed, being based on its transformation to pyruvate by lactate dehydrogenase. The reaction is forced in that direction by an excess of NAD^+ and the trapping of pyruvate by hydrazine. The resulting increase in absorbance at 340 nm due to the reduction of the NAD^+ is related to the amount of lactic acid originally present in the sample[11]. To date, several enzymatic-based assay kits are commercially available, including those for determination of acetic acid, formic acid, pyruvic acid, and ethanol[12]. Although the kits can be handled by inexperienced persons, they are very costly. In addition, a sample pretreatment is required due to interference of sugars present in bacterial cultures. In 1982, ion exchanged HPLC for determination of organic acids was established[13]. The authors were able to separate 25 acids that might be produced in bacterial cultures by using an Aminex HPX-87H column (Bio-Rad Laboratory). The 60-min time period required, however, might be longer than that of GLC analysis. Carboxylic acid analyser linked HPLC was also introduced for organic acid analysis[14]. Since a specialized column or complex analyser is required, the last two methods are not widely used.

Today, reversed phase HPLC methods are very popular in general, but not for the determination of highly polar molecules. Our aim was to introduce RP-C_{18} as a stationary phase for routine and inexpensive determination of organic acids (lactic, acetic, formic, and pyruvic acids) produced to some extent by various LAB, including *S. mutans*. Acid concentrations and molar ratios may provide information of participating glycolytic pathways if distinct a carbon source was used. This study was undertaken to attain, in a single run, the separation and quantification of the acids present in *S. mutans* cultures, and to evaluate the developed HPLC method for the determination of such.

MATERIALS AND METHODS

Reagents and chemicals

All acids and reagents used were of analytical grade. Lactic acid and sodium pyruvate were purchased from Sigma, USA. Formic acid and acetic acid were obtained from Merck, Germany. Acid stock solutions were separately prepared in water. The composite standard was prepared from samples of these stock solutions to obtain final concentrations ranging between 5.0 and 27.0 mM for acetic, and formic acids, between 4.0 and 22.0 mM for lactic acid, and 0.1–1.0 mM for pyruvic acid. With the same concentration ranges, they were diluted with liquid media to simulate the matrix effect of cultured samples. Several types of broth media were used: Brain Heart Infusion (BHI) (LabScan, Samutsakorn, Thailand), the modified APT-d (1 litre contains: casein tryptone, 12.5 g; yeast extract, 7.5 g; sodium chloride, 5 g; potassium phosphate, 5 g; sodium citrate, 5 g; polysorbate 80, 0.2 g; magnesium sulphate, 0.8 g thiaminehydrochloride, 1 mg; dextrose, 10 g), and other modified APT where glucose (APT-g), lactose (APT-l), or sucrose (APT-s) was used in place of dextrose.

HPLC method

The HPLC method was carried out using a system consisting of PU-2080 Plus Intelligent pump (Jasco, Japan), 7725i injection loop (Rheodyne, USA), UV-975 Intelligent UV/VIS detector, and Waters 745 Data Module (Millipore, USA). Separation was performed with a µRPC C_2/C_{18} column (100 x 4.6 mm) (Amersham Biosceiences, Uppsala, Sweden) at ambient temperature. The mobile phase was prepared

by mixing acetonitrile (5% v/v) and tetrabutylammonium phosphate (TBAP) monobasic (6.0 mM final concentration) (Sigma-Aldrich, Steinheim, Switzerland) in HPLC grade water, and filtered through a 0.2-μm filter before use. A flow rate of the mobile phase was 1.0 ml/min. The UV/VIS detector was fixed at a wavelength of 210 nm. Its response was adjusted to an attenuation of 64. Injection volume was 20 μl. Three standard solutions were injected each day to verify the reproducibility of the method. Identifications were based on matching retention times of standards.

Linearity, precision, accuracy, and recovery of the method

All experiments were done in triplicate. Linearity of the method was validated at five concentrations chosen in such a way that the whole expected concentration range of each acid in the sample was covered. A calibration curve was constructed by linear regression of the observed average peak area versus concentration. The coefficients of the regression curves (r) and the squares of the correlation coefficients (r^2) were calculated using the least squares method. Precision was determined by consecutive injections of sample blank and samples spiked with different acid concentrations. The average area of the samples subtracted from that of the blank, the standard deviation and the relative standard deviation (RSD) were calculated. Accuracy was evaluated from the spiked experiments as % recovery, which was calculated by dividing the measured concentration with the added concentration with the added concentration and multiplying by 100.

Growth and production of organic acids by *S. mutans*

All growth experiments were performed under anaerobic atmosphere using gas packs (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) at 37 °C. *S. mutans*, available through the American Type Cell Collection (ATCC 25175), was maintained on Trypticase soy agar (Oxoid) supplemented with 5% defibrinated horse blood (TCS Microbiology, Buckingham, United Kingdom). *S. mutans* stock culture was prepared by suspending individual colonies in 50 mM sodium phosphate (pH 7) to give an A_{550} of approximately 1.2. The suspension was used to inoculate BHI or modified APT media. After 32 h of incubation, cells were removed by centrifugation (1,100 *g*, 10 min). Proteins and residual sugars were excluded from cell-free supernatants by using Carrez reagents[15]. In brief, 5 ml of Carrez I solution (85 mM potassium hexacyanoferrate II) and 5 ml of Carrez II solution (250 mM zinc sulphate) were added to 60 ml of distilled water containing 10 ml of the cell-free broth. The pH of the solution was adjusted to 8 using 0.1 mM NaOH, and the volume was made up to 100 ml with distilled water. The solution was mixed with activated charcoal (1%), agitated, and then filtered. sample blank was done in paralled by using the fresh medium instead of the cultured medium. The clarified broth (an extracted sample) was determined for the presence of acids using the external standard method.

RESULTS AND DISCUSSION

HPLC analysis of organic acids

Separation of organic acidic compounds causes some problems in liquid chromatography, especially if the reversed-phase systems and aqueous eluents are used. These samples are scarcely retarded, but partly excluded or eluted as strongly asymmetric peaks. This problem partially arise from dissociation of these compounds, and has not been solved satisfactorily even with ion-exchange chromatography[16]. One way to circumvent the problem is to adjust the eluent's pH to suppress the dissociation. However, for small organic acids like pyruvic (pK_a 2.49), formic (pK_a 3.75), lactic (pK_a 3.86), and acetic acid (pK_a 4.76), a very low pH is required. In consequence, problems with the usual chromatographic equipment may occur. Another way is to add suitable counter-ions to the mobile phase to promote 'salt formation' with the acids. The ion pairs formed alter the retention behaviour of ionic compounds substantially. In our study, TBAP was used as the ion pairing reagent for the tested acids. Both acids and TBAP ionize completely in the mobile phase used, becoming negatively and positively charged molecules, respectively. Ionic interaction between ionized acids and tetrabutylammonium ions has thus occurred, forming neutral-like compounds. Whether the acids can be separated on the RP-column is thus dependent on the difference in their hydrophobicities[17].

Selectivity of the method

Separation of formic, lactic, acetic, and pyruvic acids on the μRPC C2/C18 column was optimized empirically. The mobile phase containing acetonitrile (5% v/v) and TBAP (6.0 mM) in water gave the best resolution at a flow rate of 1.0 ml/min. Detection at 210 nm was preferred because most acids gave the highest response at this wavelength. This optimal HPLC conditions were able to separate all tested acids

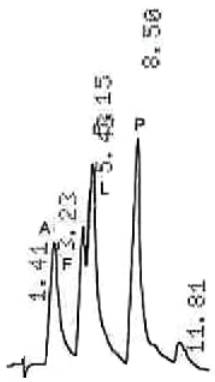

Fig. 1 HPLC chromatogram at 210 nm of standard solutions of organic acids on µRPC C2/C18 column (100 x 4.6 mm). Concentrations of test acids were: A, acetic acid (10.5 mM); F, formic acid (10.4 mM); L, lactic acid (8.05 mM); and P, pyruvic acid (0.36 mM). Mobile phase was the mixture of 5% v/v acetonitrile and 6 mM TBAP in water, and set up with the rate of 1 ml/min. Injection volume was 20 µl with signal attenuation of 64. Numbers indicate peak retention times in minutes.

(Fig. 1). Upon method validation, however, the system was found to be suited only for acetic acid, lactic acid, and pyruvic acid (Tables 1–4). A widely broad peak covering the peaks of all tested acids was observed when clarification was not conducted (data not shown), resulting that acid levels were unlikely to be determined. By using of Carrez reagents prior to the assay, in contrast, the quality of the HPLC chromatograms was highly improved (Figs 2b and 2c). Residual nutrients present following the extraction were eluted in the void volume with retention time t_R < 2 min (Fig. 2a). This procedure also extended the life of the column.

The separation of lactic acid from formic acid was poor. The resolution between them was only 0.3 under optimal conditions (Fig. 1). Any conditions leading to an enhancement of their resolution caused an increased t_R of acetic and pyruvic acids (results not shown). The best resolution was found between lactic acid and pyruvic acid (Fig. 1 and Table 1). Since pyruvic acid has a higher extinction coefficient in addition to being well resolved, this system was selective in the determination of pyruvic acid.

Linearity, precision, accuracy, and recovery of the method

For each acid, the linearity was validated at five concentrations. Calibration curves were linear for all acids tested ($r^2 > 0.9990$) (Table 2). The RSD was 0.66, for acetic acid 0.37, for lactic acid, and 0.36 for pyruvic acid, (Table 3). With the mixed standard,

Table 1. Times of elution (t_R)[a] and capacity factor (k')[b] of standard acids separated by the same conditions as in Fig.1 (N = 6)

Organic acid	HPLC parameter	
	t_R	k'
Acetic acid	3.23± 0.03	1.291
Formic acid	5.15± 0.01	2.652
Lactic acid	5.43 ± 0.01	2.851
Pyruvic acid	8.50 ± 0.02	5.028

[a] Time in minutes; [b] Number of column volumes required to elute each acid

Table 2. Coefficients of the regression curve and the square of the correlation coefficient for each organic acid (N = 6). HPLC conditions were the same as described in Fig. 1

Organic acid	Acid range (mM)	Slope	Intercept	r^2
Acetic acid	5.0– 27.0	5.8×10^4	- 77180	0.9996
Lactic acid	4.0– 22.0	6.3×10^4	- 99524	0.9997
Pyruvic acid	0.15– 1.0	3×10^6	+38993	0.9992

only about 30% of formic acid can be precisely determined. Under the conditions described, the system was therefore optimal for analyzing acetic acid, lactic acid, as well as pyruvic acid present in *S. mutans* cultures (Table 3). Less precise quantification of formic acid was mainly due to peak overlapping. Accuracy of the method was determined as the agreement between the measured concentration and the true (added) concentration. As with to the precision assay, better accuracy was found for acetic, and pyruvic acid with relative errors of 1.36, 3.49, and 1.04%, respectively (Table 4). The percentage of recovery of known concentrations of acetic, lactic, and pyruvic acids following the extraction was determined. The recovery of acetic and lactic acids was > 95 %. Pyruvic acid was less recovered (< 95 %) in comparison with acetic and lactic acid. This might be because of very low concentration range of the acid added (0.15-1.0 mM) (Table 4). In addition to the simplicity and efficiency of the clarifying method, reliable results were obtained when potential interfering compounds were selectively removed by Carrez reagents[15].

Analysis of organic acids in *S. mutans* cultures

Production of the organic acids in *S. mutans* cultures was analysed using the optimal HPLC conditions. Their presence was determined by comparing t_R of peaks found with those of known standards. Several unidentified components (U), including M(s) were apparent in the control medium (Fig. 2a). Although identity of M(s) has not been established, they were separated completely from any interesting peaks (Figs. 2b and 2c). Of other APT

control media (APT-g, APT-l, or APT-d), a similar chromatographic pattern to Fig. 2A was obtained (results not shown). It was noted that BHI might not be a suitable medium for *S. mutans* in analysing acid metabolites, because more interfering compounds were apparent following the extraction (data not shown). Reproducibility of the extraction procedure was good. The coefficients of variation between concentrations in triplicate extractions were < 0.66 (Table 3). Variation in the t_R of the acids was insignificant. The stability of the column was very good. More than 200 samples were separated on the column when the chromatograms of Fig. 2 were run. Separation characteristics were the same as those obtained from a new column (results not shown). As expected, acetic acid and formic acid were not produced by *S. mutans* grown in all tested media since the principal pathway was the production of lactic acid (Fig. 2B). Accordingly, the developed method was more precise in measuring lactic acid. A small amount of acetic acid (4.93 mM) was generated by *Lactobacillus jensenii* 5L08, a microorganism of vaginal origin (Fig. 2C). This result partly indicated that a sugar-metabolic shift occurred by the action of this strain (unpublished data). Additional information on its mucous metabolism is being examined in our laboratory.

CONCLUSION

A simple, fast, and isocratic separation of fermentation end products on an inexpensive stationary phase, C_{18} column, was developed. About 10-min time period was required for a complete separation, being shorter than those analyzed by GLC and ion-exchange HPLC. No additional interference was apparent when bacterial samples were purified before performing the chromatography. All the acid products of *S. mutans* could be separated in just one run by the HPLC method. The simplicity and rapidity of the procedure justify the routine use of this method in various fields of bacteriology.

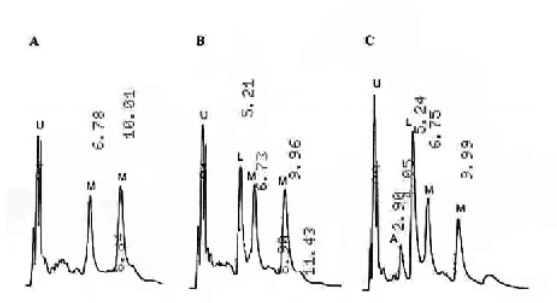

Fig. 2 HPLC chromatograms of (A), the extracted APT-s broth; (B), the extracted supernatant of *S. mutans* 25175; and (C), the extracted supernatant of *Lactobacillus jensenii* 5L08 18. Both bacterial strains were grown in APT-s under anaerobic conditions at 37 °C for 48 h. U, unidentified components; A, acetic acid; L, lactic acid; M(s), APT-s medium components. HPLC conditions were the same as described in Fig. 1. Numbers indicated retention times in minute of the peaks

Table 3. Precision of acetic, lactic, and pyruvic acids determined by spicing the acids in the modified APT-s (N = 6). Chromatographic conditions were as in Fig. 1

Acetic acid (m M)	RSD	Lactic acid (m M)	RSD	Pyruvic acid (m M)	RSD
5.23	0.66[a]	4.03	0.26	0.18	0.32
15.70	0.10	12.07	0.37[b]	0.54	0.14
26.15	0.16	21.30	0.32	0.90	0.36[c]

[a, b, c] The highest RSD of each acid indicated in Results and Discussion

Table 4. Accuracy and percentage recovery of acetic, lactic, and pyruvic acid determined by spicing APT-s with known concentrations of acids (N = 3). Chromatographic conditions were as in Fig. 1

Acids	Conc. added (mM)	Conc. measured (mM)	% Recovery	Relative error (RE)
Acetic acid	5.23	4.10	78.39	-6.29
	15.70	15.42	98.22	-4.70
	26.15	25.29	96.71[a]	-1.36[a]
Lactic acid	4.03	3.65	90.57	-19.72
	12.07	11.13	92.21	-11.90
	21.30	20.29	95.25[b]	-3.49[b]
Pyruvic acid	0.18	0.13	73.22	-1.16
	0.54	0.45	83.33	-1.78
	0.90	0.82	91.11[c]	-1.04[c]

[a, b, c] Data indicated in Results and Discussion

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