Journal of Chromatographic Science 2012;50:709–713
doi:10.1093/chromsci/bms046 Advance Access publication May 25, 2012

Article

Ion-Exclusion Chromatography Determination of Organic Acid in Uridine 5′-Monophosphate Fermentation Broth

Huanqing Niu†, Yong Chen†, Jingjing Xie, Xiaochun Chen, Jianxin Bai, Jinglan Wu, Dong Liu and Hanjie Ying*

State Key Laboratory of Materials-Oriented Chemical Engineering, College of Life Science and Pharmaceutical Engineering, Nanjing University of Technology, Nanjing 210009, People's Republic of China

*Author to whom correspondence should be addressed. Nanjing University of Technology, No. 30, Puzhu South Road, Distict of Pukou, Nanjing 211816, Jingsu Province, China. Email: yinghanjie@njut.edu.cn

†These authors contributed equally to this study.

Received 21 June 2011; revised 14 September 2011

Simultaneous determination of organic acids using ion-exclusion liquid chromatography and ultraviolet detection is described. The chromatographic conditions are optimized when an Aminex HPX-87H column (300 × 7.8 mm) is employed, with a solution of 3 mmol/L sulfuric acid as eluent, a flow rate of 0.4 mL/min and a column temperature of 60°C. Eight organic acids (including orotic acid, α-ketoglutaric acid, citric acid, pyruvic acid, malic acid, succinic acid, lactic acid and acetic acid) and one nucleotide are successfully quantified. The calibration curves for these analytes are linear, with correlation coefficients exceeding 0.999. The average recovery of organic acids is in the range of 97.6% ∼ 103.1%, and the relative standard deviation is in the range of 0.037% ∼ 0.38%. The method is subsequently applied to obtain organic acid profiles of uridine 5′-monophosphate culture broth fermented from orotic acid by *Saccharomyces cerevisiae*. These data demonstrate the quantitative accuracy for nucleotide fermentation mixtures, and suggest that the method may also be applicable to other biological samples.

Introduction

Nucleotides are widely used as taste-enhancing food additives and pharmaceutical intermediates. They are also a significant material in the synthesis of carbohydrates, which are of intense interest in various scientific fields (1). Nucleotides can be made by either a fermentative process or a biocatalytic process. Because the pathway from raw materials to product is long and complicated, whole-cell biotransformation has been exploited for large-scale nucleotide production (2).

Recently, cellular metabolism and flux analysis have been studied extensively. These studies require quantitative analysis of the compositional constituents found in the cellular medium (3). During biocatalytic processes, numerous short-chain carboxylic acids are produced in liquid media (4). These important intermediate metabolites formed during the Embden–Meyerhof–Parnas (EMP) pathway and the tricarboxylic acid (TCA) cycle delineate a precise metabolic pathway in the microorganism.

Organic acids have been determined by many analytical techniques. Non-enzymatic spectrophotometric and enzymatic methods to determine organic acid individually have been described in the literature, but these methods are tedious and time-consuming. At present, chromatographic and electrophoretic methods are being applied to simultaneously identify and quantify organic acids (5, 6). These include thin-layer chromatography (6, 7), reversed-phase high-performance liquid chromatography (HPLC) (8), ion-exchange chromatography (9), gas chromatography (10) and capillary electrophoresis (11). Although thin-layer chromatography is a rapid, simple and economic method, it has the disadvantages of a long deployment time and poor separation of components. Reversed-phase and ion-exchange HPLC with ultraviolet (UV) spectrophotometric detection have been the most widely used methods for high column selectivity and detector sensitivity, but they require complex sample preparation procedures (12). Gas chromatography is available for the determination of organic acid, but it is limited by its cost and the complexity of both the instruments and sample preparation (13, 14). Capillary electrophoresis has also been used for high resolution, simplicity, automation, short analysis times, low consumption of reagents and samples and minimum preparation of sample; however, it suffers from lower reproducibility than enzymatic and chromatographic methods (6, 14). Moreover, most of the methods mentioned previously require large amounts of organic solvents and reagents that are hazardous to the environment and human health (13). Therefore, a simple and environmentally friendly method for determining organic acids remains an analytical challenge.

In the present study, we demonstrate an ion-exclusion chromatographic method with a UV detector for the determination of eight organic acids and one nucleotide. Then, we successfully profile the organic acids produced by *Saccharomyces cerevisiae* from orotic acid in the culture broth. The aim of our work is to develop a simple and reproducible ion-exclusion chromatographic method to analyze the major organic acids that are produced during the biocatalysis process. Our procedure offers an advantage in speed and selectivity, and it can also be applied to the analysis fermentation broth samples of other nucleotides.

Experimental

Instrumentation

The Agilent 1200 series HPLC system was equipped with an isocratic pump (G1311A), a column heater (G1316A), a variable wavelength detector (G1314B) and an autoinjector (G1329A). A Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm i.d.) was used for analysis. The analytic column was

packed with sulfonated styrene divinylbenzene resin in the H^+ form (9 μm particle size).

For ion-exclusion, samples were injected via a 20-μL loop, eluted at a flow rate of 0.4 mL/min and detected at 210 nm. Sulfuric acid at different concentrations was tested as the eluent, ranging from 1 to 20 mmol/L. Different column temperatures, ranging from 20 to 60°C, were also investigated.

Reagents and solutions

The chemicals for the standard solutions were purchased from Sigma (St. Louis, MO). The following stock solutions were prepared: 0.1 g/L UMP, 0.1 g/L orotic acid, 0.1 g/L α-ketoglutaric acid, 0.1 g/L pyruvic acid, 1 g/L malic acid, 1 g/L succinic acid, 1 g/L lactic acid, 1 g/L acetic acid and 1 g/L citric acid.

Microorganism, medium and culture conditions

The strain, *Saccharomyces cerevisiae* As2.398(2n), was stored in our laboratory. The seed culture medium contained (g/L): yeast extract 10, peptone 20 and glucose 20. The prepared seed culture was inoculated (5%, *v*/*v*) into a 1-L shake flask containing 300 mL fermentation medium. The fermentation medium contained (g/L): glucose 50, peptone 5, yeast extract 2, $(NH_4)2HPO_4$ 2, $MgSO_4.7H_2O$ 1, KH_2PO_4 2. The initial pH values of the seed medium and fermentation medium were adjusted to 5.8 and 8.0, respectively, by addition of 1.0 mol/L NaOH and 0.1 mol/L HCl. Cultivation was carried out at 30°C for 48 h.

Aliquot of the culture was centrifuged (Centrifuge 5804R, Eppendorf, Hamburg, Germany) at 8,000 rpm at 4°C for 10 min and the supernatant was analyzed. Standards from the stock solution and fermentation samples were diluted with deionized water and filtered through 0.45-μm nylon Acrodisc filters (Gelman Science, Ann Arbor, MI) before injection.

Results and Discussion

The determination of organic acids by ion-exclusion chromatography has been reported. Typically, resin-based polystyrene-divinylbenzene columns in the H^+ form are used, combined with aqueous solutions of sulfuric acid as eluent (15–18). Ion-exclusion chromatography is based on a mechanism suggesting that the sulfonic groups are mostly fixed on the surface of the polystyrene-divinylbenzene resin and form a negatively charged shield on the polymeric surface, which is often termed the Donnan membrane (19). This membrane enables the non-ionic species to penetrate the resin and almost completely excludes the charged species. When compounds enter the column, the ones with dissociated protons are repelled by electrostatic repulsion from the Donnan membrane, whereas the undissociated compounds penetrate the membrane and interact with the stationary phase. Therefore, the hydrophobic interactions between the resin and the compounds, and the degree of access to the pore, determine the ability of the column to separate the compounds. The elution of the organic acids is approximately in the order of ascending pKa values. Strong acids, due to the low pKa, are almost totally ionized and eluted near the column. A higher pKa means less dissociation of the organic acid, and this leads to better separation on ion-exclusion columns. In addition, other variables, including van der Waals forces, hydrogen bonding and the hydrophobicity of the acids, may modify the elution order to some extent.

Effect of eluent concentration and column temperature

During determination of these organic acids, several parameters may have an impact on the resolution and retention times. These parameters include eluent strength, eluent pH, column temperature and flow rate. Here we show that the strength of the eluent acid and the column temperature are

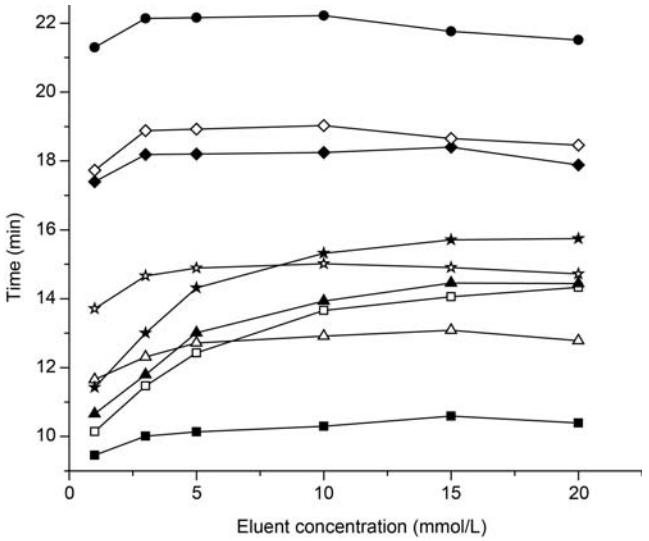

Figure 1. Effect of eluent acid strength on retention times of organic acids. Black square, UMP; white square, orotic acid; black triangle, α-ketoglutaric acid; white triangle, citric acid; black star, pyruvic acid; white star, malic acid; black diamond, succinic acid; white diamond, lactic acid; black circle, acetic acid.

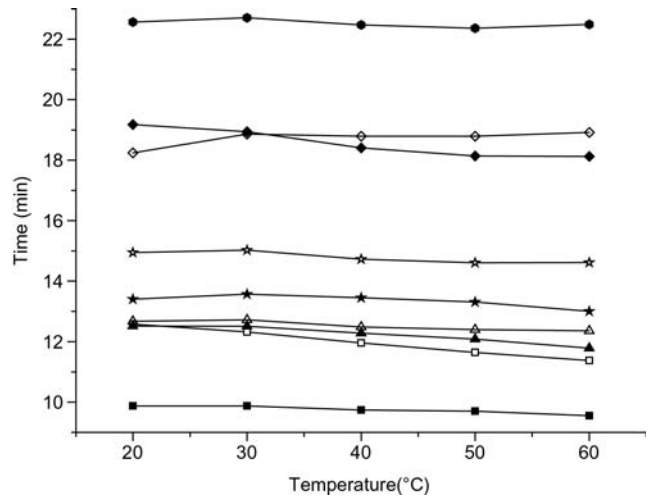

Figure 2. Effects of column temperature on retention times of organic acids. Black square, UMP; white square, orotic acid; black triangle, α-ketoglutaric acid; white triangle, citric acid; black star, pyruvic acid; white star, malic acid; black diamond, succinic acid; white diamond, lactic acid; black circle, acetic acid.

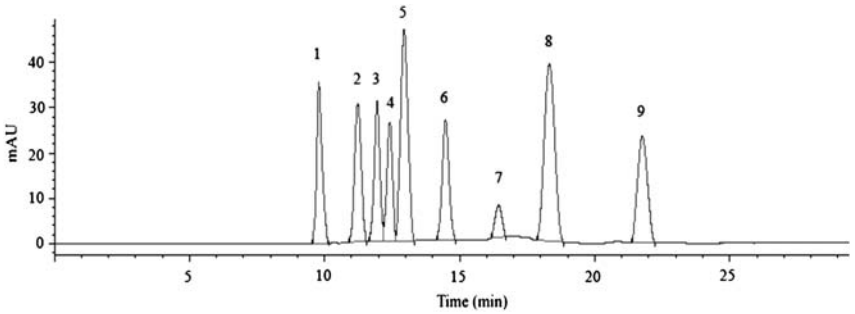

Figure 3. Chromatogram of a standard solution of organic acids. Isocratic elution using an Aminex HPX-87H (300 × 7.8 mm i.d.). Aqueous sulphuric acid solution, 3 mM; flow rate, 0.4 mL/min; temperature, 60°C; detection, UV absorption at 210 nm. Peaks: 1, UMP; 2, orotic acid; 3, α-ketoglutaric acid; 4, citric acid; 5, pyruvic acid; 6, malic acid; 7, succinic acid; 8, lactic acid; 9, acetic acid.

considered to be two major factors in the optimization process of the chromatographic conditions.

According to Chinnici *et al.* (18), either sulfuric acid or phosphoric acid can be used as the mobile phase to analyze organic acids in Aminex HPX-87H columns. In our study, sulfuric acid was selected as the eluent for higher resolution and safety of the chromatographic column. Sulfuric acid at different concentrations was tested as the eluent, ranging from 1 to 20 mmol/L. The result is shown in Figure 1. Due to its high pKa, acetic acid is almost undissociated and has the longest retention time, so it could easily be separated from the other organic acids. Its retention time was slightly influenced by the changing concentration of the eluent, while succinic acid, lactic acid and UMP also achieved baseline separation from the other tested compounds. The retention times of orotic acid, α-ketoglutaric acid and pyruvic acid and the eluent acid strength are highly positively correlated. It seems that these three organic acids, and citric acid and malic acid, are hard to separate. Finally, we chose an eluent concentration of 3 mmol/L, at which the five organic acids were better separated than separations achieved at other concentrations (Figure 1).

The effect of column temperature was also investigated, and the result is shown in Figure 2. As the column temperature increased, the retention time of the organic acid decreased, but weakly. The reason for this is that high temperature can optimize the efficiency of the chromatography by minimizing the band spreading, because of the slow mass transfer in the stationary phase and the decrease in the backpressure of the column. Thus, a column temperature of 60°C was selected to develop the ion exclusion HPLC method.

Because slow mass transfer in the stationary phase contributes to band broadening, low flow rates are typically required for efficient operation. However, low flow rates will increase the analysis time. The flow rate for the Aminex HPX-87H is usually 0.6 mL/min. A flow rate of 0.4 mL/min was chosen to increase efficiency. The output under these conditions is presented in Figure 3.

Linearity of calibration curves, reproducibility, limit of detection and recovery

The peak area under absorbance detection at 210 nm was studied for each organic acid. Table I presents calibration data for eight organic acids and UMP. Five levels of concentration were tested in triplicate, and a calibration curve for each acid was calculated by regressing the peak area against the corresponding acid concentration. Excellent linearity was obtained, with correlation coefficients ranging from 0.999 to 1.000. The limit of detection (LOD) of each of the compounds was calculated considering a signal-to-noise ratio (S/N) of 3. The instrument precision was verified by calculating the relative standard deviation (RSD) of the retention times from five successive injections, ranging from 0.037 to 0.38, which was acceptable for our analytical purposes.

The accuracy of the method was verified by the technique of standard addition. This was performed by spiking the application sample obtained during homogeneous catalysis with a known concentration within the calibration range of the individual organic acid being analyzed. Recoveries of various organic acids in the broth ranged from 97.6 to 103.1%. Therefore, we conclude that this method has a high selectivity and reproducibility, and this is helpful in the accurate and simultaneous quantification of organic acids.

Table I
Analytical Characteristics at 3 mM Sulphuric Acid and 60°C

Organic acid	R of calibration curve	SD (mg/L)	RSD (%) ($n = 5$)	Linear range (mg/L)	LOD (S/N = 3) (mg/L)	Recovery (%) ($n = 5$)
UMP	0.9991	0.19	0.19	200–1,000	1.6	101.5
Orotic acid	0.9996	0.17	0.17	50–500	1.2	97.6
α-Ketoglutaric acid	0.9992	0.20	0.20	50–1,000	1.0	102.2
Citric acid	0.9999	0.78	0.078	50–1,000	7.0	100.2
Pyruvic acid	0.9994	0.044	0.044	25–1,000	0.6	98.0
Malic acid	0.9999	1.3	0.13	50–1,000	10.2	103.1
Succinic acid	1.0000	3.8	0.38	60–1,000	18.3	97.0
Lactic acid	0.9997	0.37	0.037	60–1,000	16.1	99.5
Acetic acid	1.0000	3.0	0.30	100–1,000	29.3	99.3

Table II
Concentration Change of Organic Acids during the Fermentation Processes

Time (h)	Orotic acid (g/L)	Citric acid (g/L)	Pyruvic acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Acetic acid (g/L)
12	0.06	0	0	1.21	2.46	0.47
24	0.11	0.25	0	3.53	8.13	1.60
32	0.23	0.34	0.42	4.69	10.23	2.01
48	0.28	0.45	1.23	2.34	1.54	2.57

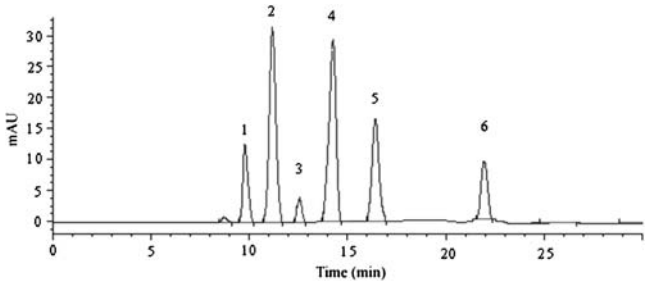

Figure 4. Concentration of organic acids in the biocatalytic reaction of UMP production at 24 h. Peaks: 1, UMP; 2, orotic acid; 3, citric acid; 4, malic acid; 5, succinic acid; 6, acetic acid.

Application to UMP fermentation broth

The method was applied to determine the sample composition at different times during the biocatalytic process. The organic acids were quantified using external calibrations based on peak area. Samples were diluted 50 times before microfiltration and injection, and each sample was injected in triplicate. The results are presented in Table II and Figure 4 shows a chromatogram of the sample at 24 h.

The results show that six organic acids, orotic acid, citric acid, pyruvic acid, malic acid, succinic acid and acetic acid, could be detected during the fermentation process. The malic acid and succinic acid accumulate to a certain amount at the intermediate stage, and most of the acids are consumed as carbon sources at the end of the reaction. At 24 h, the production of UMP achieved its maximum value (8.34 g/L) and pyruvic acid had not yet formed. The accumulation of organic acids influences the production of UMP because of acid toxicity to proteins and the wastage of carbon. The change in the concentration of organic acids can reflect the changes in the EMP pathway and the TCA cycle. These results are helpful for breeding industrial microorganisms through genetic engineering and enhancing the production of UMP by inhibiting the flux of the EMP pathway and the TCA cycle.

Conclusions

In this work, we have proposed an ion-exclusion chromatographic method with a resin-based Aminex HPX-87H column for the quantification of eight organic acids and one nucleotide. The advantages of this method are the simple preparation of samples, the easy and rapid operation of the instrument, the high precision and accuracy of the results, and the inexpensive and environmentally friendly materials and reagents needed for the analysis. Finally, this method is useful for analyzing organic acids in UMP fermentation broth, and it can also be applied in the routine analysis of other systems that have these analytes.

Acknowledgments

This work was supported by a grant from National Outstanding Youth Foundation of China (Grant No.: 21025625); National Key Technology RD Program (Grant No. 2008BAI63B07); National Basic Research Program of China (973 Program, 2009CB724700); National High-Tech Research and Development Program of China (863) (Grant Nos.: 2010AA101502, 2007AA100404), National Natural Science Foundation of China, Youth Program (Grant No.: 21006051), Changjiang Scholars and Innovative Research Team in University (PCSIRT), and Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

References

1. Wang, X., Wang, X.W., Yin, M.X., Xiao, Z.J., Ma, C.Q., Lin, Z.X., *et al.*; Production of uridine 5'-monophosphate by *Corynebacterium ammoniagenes* ATCC 6872 using a statistically improved biocatalytic process; *Applied Microbiology and Biotechnology*, (2007); 76: 321–328.
2. Chen, Y., Li, S.Y., Xiong, J., Li, Z.J., Bai, J.X., Zhang, L., *et al.*; The mechanisms of citrate on regulating the distribution of carbon flux in the biosynthesis of uridine 5'-monophosphate by *Saccharomyces cerevisiae*; *Applied Microbiology and Biotechnology*, (2010); 86: 75–81.
3. Geng, X.M., Zhang, S.F., Wang, Q., Zhao, Z.B.(K.); Determination of organic acids in the presence of inorganic anions by ion chromatography with suppressed conductivity detection; *Journal of Chromatography A*, (2008); 1192: 187–190.
4. Eiteman, M.A., Chastain, M.J.; Optimization of the ion-exchange analysis of organic acids from fermentation; *Analytica Chimica Acta*, (1997); 338: 69–75.
5. Suárez-Luque, S., Mato, I., Huidobro, J.F., Simal-Lozano, J., Sancho, M.T.; Rapid determination of minority organic acids in honey by high-performance liquid chromatography; *Journal of Chromatography A*, (2002); 955: 207–214.
6. Li, Y.H., Huang, B.X., Shan, X.Q.; Determination of low molecular weight organic acids in soil, plants, and water by capillary zone electrophoresis; *Analytical and Bioanalytical Chemistry*, (2003); 375: 775–780.
7. Tang, Y.J., Wu, M.J.; The simultaneous separation and determination of five organic acids in food by capillary electrophoresis; *Food Chemistry*, (2007); 103: 243–248.
8. Destandau, E., Viala, J., Jardy, A., Hennion, M.C., Bonnet, D., Lancelin, P.; Robustness study of a reversed-phase liquid chromatographic method for the analysis of carboxylic acids in industrial reaction mixtures; *Analytica Chimica Acta*, (2006); 572: 102–112.
9. Lues, J.F.R., Botha, W.C., Smit, E.J.; Ion-exchange HPLC analysis of a broad spectrum of organic acids from matured Cheddar cheese and assessment of extraction methods; *Food Research International*, (1998); 31: 441–447.
10. Xiao, J.B.; Identification of organic acids and quantification of dicarboxylic acids in bayer process liquors by GC–MS; *Chromatographia*, (2007); 65: 185–190.
11. Peres, R.G., Moraes, E.P., Micke, G.A., Tonin, F.G., Tavares, M.F.M., Rodriguez-Amaya, D.B.; Rapid method for the determination of organic acids in wine by capillary electrophoresis with indirect UV detection; *Food Control*, (2009); 20: 548–552.
12. Destandau, E., Vial, J., Jardy, A., Hennion, M.C., Bonnet, D., Lancelin, P.; Development and validation of a reversed-phase liquid chromatography method for the quantitative determination of carboxylic acids in industrial reaction mixtures; *Journal of Chromatography A*, (2005); 1088: 49–56.
13. Dias, J.C., Suzuki, E., de Albuquerque, C.L., Ferreira, A.L., Brito, A.R.M.S., Kubota, L.T.; Determination of short-chain fatty acids in dietary fiber extracts using ion-exclusion chromatography with suppressed conductivity detection; *Journal of Pharmaceutical and Biomedical Analysis*, (2009); 49: 1128–1132.
14. Mato, I., Suárez-Luque, S., Huidobro, J.F.; A review of the analytical methods to determine organic acids in grape juices and wines; *Food Research International*, (2005); 38: 1175–1188.

15. Morales, M.L., Gonzalez, A.G., Troncoso, A.M.; Ion-exclusion chromatographic determination of organic acids in vinegars; *Journal of Chromatography A*, (1998); 822: 45–51.
16. Chinnici, F., Spinabelli, U., Amati, A.; Simultaneous determination of organic acids, sugars, and alcohols in musts and wines by an improved ion-exchange HPLC method; *Journal of Liquid Chromatography and Related Technologies*, (2002); 25: 2551–2560.
17. Chinnici, F., Spinabelli, U., Riponi, C., Amati, A.; Optimization of the determination of organic acids and sugars in fruit juices by ion-exclusion liquid chromatography; *Journal of Food Composition and Analysis*, (2005); 18: 121–130.
18. Papadakis, E.N., Polychroniadou, A.; Application of a microwave-assisted extraction method for the extraction of organic acids from Greek cheeses and sheep milk yoghurt and subsequent analysis by ion-exclusion liquid chromatography; *International Dairy Journal*, (2005);15: 165–172.
19. Novic, M., Haddad, P.R.; Analyte-stationary phase interactions in ion-exclusion chromatography; *Journal of Chromatography A*, (2006); 1118: 19–28.