0273-2289/95/5152—0519$05.40

A Novel Method for Isolating and Analyzing Organic Acids in Biological Cultures

Scientific Note

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Index Entries: HPLC; organic acids; Polypore H; PRP-X300

INTRODUCTION

Fermentatively produced organic acids have significant potential as chemical feedstocks for the production of various commodity materials (*1,2*). Such acids include acetic and succinic acids. Fermentations frequently result in the simultaneous production of two or more organic acids, and often other fermentation products as well (*3,4*). This necessitates separation of these products from each other, so that quantification and purification can be achieved. A multitude of methodologies for the identification, purification, and quantitation of organic acids has been developed and described; both liquid and gas chromatography have been used for such separations (*5–8*). High-performance liquid chromatography (HPLC) media used for the separation of organic acids have included C18 columns, Aminex HPX-87H (ion-moderated partition resin), TEAP-Si 100 Polyol (strongly basic anion-exchange resin), Dowex 1 (cation-exchange column), Shodex Ionpak KC811, and others (*9–17*). Methodologies for HPLC analysis of organic acids also vary in these aspects:

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1. Sample pretreatment (e.g., pretreatment with Sep-Pak C18 cartridges or with DEAE-Sephadex);
2. Mobile-phase composition (e.g., dilute sulfuric acid or formic acid); and
3. Method of organic acid detection (e.g., refractive index or light absorption) (*12,14,18–21*).

In this study, we present a methodology for isolating and quantifying organic acids found in fermentation broths. The methodology is simple, utilizes dual separation chemistries to effect an enhanced separation capacity, and is durable in terms of HPLC column life.

METHODS

HPLC Methodology

Organic acid standards were prepared as 0.01–10% (w/v) aqueous solutions of the following sodium salts of organic acids: disodium malate, sodium succinate hexahydrate, sodium lactate, sodium formate, sodium acetate trihydrate, sodium propionate, and sodium citrate dihydrate. Standards (750 μL) were mixed with 250 μL 4*N* H_2SO_4, left sitting for 15–30 min, and then filtered (0.2 μm, Acrodisc HT Tuffryn, Gelman, Ann Arbor, MI). Filtrates (5 μL) were injected into three HPLC systems:

1. Polypore H column, 220 × 4.6 mm (Applied Biosystems, San Jose, CA); 0.01*N* H_2SO_4 mobile phase, 0.1–0.15 mL/min;
2. PRP-X300 column, 150 × 4.1 mm (Hamilton, Reno, NV); 0.001*N* H_2SO_4 mobile phase, 0.2 mL/min; and
3. A dual-column system consisting of a Polypore H column linked to a PRP-X300 column by 21 cm of 0.010-in. internal diameter poly ether ether ketone tubing (the sample entered the Polypore H column first); 0.01*N* H_2SO_4 mobile phase, 0.2–0.35 mL/min.

Absorbance of eluant was measured at 210 nm.

Growth Media Tested

Three complex microbiological growth media were tested for compatibility with the dual-column HPLC system. The first was a medium designed to enrich for succinic acid-producing microorganisms that contained (g/100 mL): KH_2PO_4 (0.2), $MgSO_4 \cdot 7H_2O$ (0.2), $(NH_4)_2SO_4$ (0.1), yeast extract (0.3), dextrose (20), $Na_2CO_3 \cdot 10H_2O$ (14.3), and 2(-*N*-morpholino)ethanesulfonic acid (19.5). Succinic acid-producing microorganisms were grown in this medium, and culture fluids were analyzed for succinic acid content. The second medium was designed for lactate-utilizing sulfate-reducing bacteria. It contained (g/100 mL): K_2HPO_4 (0.05), NH_4Cl

(0.1), $CaCl_2 \cdot 2H_2O$ (0.01), $MgCl_2 \cdot 6H_2O$ (0.05), sodium lactate (0.25), yeast extract (0.01), $Na_2SeO_3 \cdot 2H_2O$ (0.0000006), $Na_2WO_4 \cdot 2H_2O$ (0.0000008), sodium thioglycollate (0.1), ascorbic acid (0.1), $FeSO_4 \cdot 7H_2O$ (0.01), and Na_2SO_4 (0.1). Lactic acid disappearance was analyzed for cultures of sulfate-reducing bacteria grown with this medium. The third medium was designed for osmotolerant acetate-utilizing nitrate reducers. It contained (g/100 mL): $NaNO_3$ (2.6), $Na_3PO_4 \cdot 12H_2O$ (9.5), $NaNO_2$ (0.1), Na_2SO_4 (4.8), $NaHCO_3$ (13.6), KNO_3 (0.1), NaF (0.1), NaOH (5.1) K_2HPO_4 (0.4), and acetic acid (0.4). Acetic acid levels were tested for cultures using this medium. HPLC analyses for all three media were performed as described above for organic acid standards.

RESULTS AND DISCUSSION

Chromatographic data for organic acid analyses performed with Polypore H or PRP-X300 columns alone indicate that succinic and lactic acids are not separated by Polypore H, and malic and formic acids are not separated by PRP-X300 (Table 1). The dual-column system that utilized both of these columns in series was able to separate all of the acids tested (Table 1). At the fastest flow rate possible for the dual-column system (owing to the pressure limit of the Polypore H column), retention times ranged from 11 to 28 min. These analyses were performed at room temperature (22°C); significantly faster retention times should be possible using increased column temperatures (22). Propionic acid showed a long retention time with the dual-column system. This appears to be the result of the PRP-X300 column, since propionic acid was easily analyzed with the Polypore H column alone. Consequently, analysis of this acid by the dual-column methodology is undesirable; however, there may be some applicability of the system to purification technology for propionic acid. Similarly, there may be application of this method in succinic acid purification technology, since succinic acid elutes at a substantially remote retention time compared to other acids (Table 1). It is likely that the dual-column system enhances the separability of other fermentation products, such as alcohols.

Utilizing the dual-column system, excellent standard curves were prepared for five of the organic acids tested (Table 2). The range of concentrations analyzed was very broad (1000-fold). A standard curve for citric acid was not prepared; however, it is likely that quantitation of similar quality is possible for this acid, using the dual-column system.

Although both Polypore H and PRP-X300 columns are based on styrene divinyl benzene solid supports, they are derivatized differently to the extent that, as shown here, they exhibit significantly different properties with respect to the separation of organic acids (23,24). This study indicates that the combination of these properties can be advantageous.

Analyses of succinic, lactic, and acetic acids in three complex growth media indicated that the dual-column method was compatible with a

Table 1
Chromatographic Data from Analyses of Organic Acids Using Three Different HPLC Systems

	Acids analyzed separately[1]				Acids analyzed as a mixture[2]			
Column	Flow rate, mL/min	Acid	Retention time, min	Peak area, A_{210}·s × 10^{-3}	Flow rate, mL/min	Acid	Retention time, min	Peak area, A_{210}·s × 10^{-3}
Polypore H	0.15[3]	Malic	13.80	65036	0.1[3]	Succinic	24.14	—
		Succinic	16.03	27942		Lactic	24.14	—
		Lactic	16.10	97597				
		Formic	17.49	73642				
		Acetic	18.34	27205				
		Propionic	20.79	54284				
PRP-X300	0.20[4]	Formic	11.42	61591	0.20[4]	Formic	11.84	—
		Malic	11.60	62204		Malic	11.84	—
		Citric	13.90	91730		Citric	13.45	13833
		Lactic	14.33	90510		Lactic	15.52	18982
		Acetic	20.30	22900		Acetic	22.38	4593
		Succinic	29.54	27200		Succinic	31.57	6100
Polyore H +PRP-X300	0.20[3]	Malic	20.01	48840	0.20[3]	Malic	20.31	8354
		Formic	22.74	56653		Formic	23.04	9671
		Lactic	25.70	75750		Citric	24.64	8338
		Acetic	33.99	20981		Lactic	26.55	16275
		Succinic	43.52	22164		Acetic	35.00	3654
		Propionic	105.30	28791		Succinic	47.08	3686
	0.35[3]	Malic	11.35	28757	0.35[3]	Malic	11.53	5273
		Formic	12.91	33093		Formic	13.11	7049
		Citric	13.52	33729		Lactic	15.20	9102
		Lactic	14.55	43792		Acetic	20.18	2444
		Acetic	19.19	11993		Succinic	27.05	2566
		Succinic	25.02	12409				

[1]Acids were 10% (w/v) aqueous solutions of sodium salts of organic acids, treated as described in Methods.

[2]Mixtures were aqueous solutions of sodium salts of organic acids that contained the following concentrations (w/v): 5% sodium succinate, 5% sodium lactate (Polypore H); 1.7% sodium salt for each acid listed (PRP-X300); 2% sodium salt for each acid listed (dual column, 0.2 mL/min); 1.7% sodium salt for each acid listed (dual column, 0.35 mL/min).

[3]Mobile phase was 0.01*N* H_2SO_4.

[4]Mobile phase was 0.001*N* H_2SO_4.

Table 2
Chromatographic Data from Analyses of Different Concentrations of Organic Acids Using the Dual-Column Method[1]

Acid	Concentration, % w/v[2]	Retention time, min	Peak area, A_{210}·s × 10^{-3}	r[3]
Malic	10	20.01	48840	
	1	20.35	4789	
	0.1	20.44	462	
	0.01	20.45	99	0.9999
Formic	10	22.74	56653	
	1	23.19	5814	
	0.1	23.30	580	
	0.01	23.31	58	0.9999
Lactic	10	25.70	75750	
	1	27.14	7643	
	0.1	27.54	765	
	0.01	27.68	82	0.9999
Acetic	10	33.99	20981	
	1	36.01	2189	
	0.1	36.54	223	
	0.01	36.52	20	0.9999
Succinic	10	43.52	22164	
	1	47.97	2216	
	0.1	49.53	196	
	0.01	50.01	5	0.9999

[1]Mobile phase was 0.01*N* H_2SO_4, 0.2 mL/min.
[2]Acids were 0.01–10% (w/v) aqueous solutions of sodium salts of organic acids, treated as described in Methods.
[3] Correlation coefficient derived from linear regression curve for 0.01–10% standards.

variety of medium components (media recipes in Methods, data not shown). All three acids were easily quantitated when present in these media, utilizing the dual-column method (data not shown). So far, 75 analyses have been performed with the same column system without significant decrease in chromatographic quality.

In conclusion, results presented here provide a novel method for separating fermentation products, either for qualitative and quantitative analysis or for purification purposes. A survey of the literature concerning separation of organic acids by HPLC indicates that previous methods are either better or worse than this one, depending on an investigator's particular objectives (*5–21*). Our method is advantageous because:

1. Utilization of two different separation chemistries results in enhanced separation of organic compounds;
2. Sample preparation is an easy process that does not generate hazardous solvent waste (*see* Methods);

3. A broad range of product concentrations can be analyzed without need for dilution of the original sample;
4. The method is compatible with a variety of growth medium components; and
5. The system is durable.

ACKNOWLEDGMENTS

Thanks are given to INEL employees Sandy Fox (for HPLC advice), Greg Bala (for providing the HPLC system), Earl Johnson (for providing nitate reducer culture samples), and Joni Barnes (for providing sulfate reducer culture samples). Funding for the work described herein was provided by the United States Department of Energy (Office of Industrial Technologies; Office of Conservation and Renewable Energy; contract no. DE-AC07-76ID01570).

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