Development of a quantitative method for organic acid in wine and beer using high performance liquid chromatography

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Abstract This study was conducted to compare the organic acid content in liquors (red wine, white wine, and beer) using three different high-performance liquid chromatography analysis methods. Post-column reaction methods (method 2 and 3) were found to be more promising than UV-detection method (method 1). Using method 2 (two columns), the analyzed red wine was found to contain 2,652.4 mg/L tartaric acid and 1,392.9 mg/L lactic acid but relatively lower amounts of malic acid (271.0 mg/L). Furthermore, tartaric acid (1,160.8–2,749.1 mg/L) and malic acid (470.2–3,107.9 mg/L) were the major components in white wine. Beers were analyzed using method 3 (one column). In the analyzed foreign beers, lactic acid (95.9–226.4 mg/L), malic acid (62.2–110.5 mg/L), acetic acid (93.5–183.8 mg/L), and succinic acid (37.0–56.2 mg/L) were detected. Similar to the foreign beers, the contents of succinic acid in the domestic beers were the lowest. The proposed methods could be useful for quantitative analysis of organic acids in wine and beer.

Keywords: organic acids, UV-detection, post-column reaction, wine, beer

Introduction

In the course of liquor production, various materials such as flavor components, alcohol, and organic acids are produced. Among them, organic acids play important roles in the liquors. Firstly, organic acids are factors that impart an acidic taste to the foods, resulting in different flavors and tastes. For example, citric acid affords a fresh feeling and a smooth sour taste, whereas malic acid affords a soft sour taste. Thus, the taste of the same type of liquor can vary according to the composition of the organic acids. Moreover, organic acids provide a suitable pH for liquor fermentation and restrict the propagation of microorganisms, thereby enhancing the stability of the liquor (1).

It has been known that wine making process is divided into the following stages: alcoholic fermentation, malo-lactic fermentation (MLF), and aging process. The fermentation stage can be regulated on the basis of the contents of certain organic acids, thereby determining the quality of the final wine. During the MLF stage, wherein malic acid is converted to lactic acid, the acidity is decreased, which sometimes improves the flavor of the wine (2–4). In liquor fermentation, synthesis of citrate can be a critical factor that determines fermentation degree, monitoring the degree of metabolism of anaerobic yeast (1). Therefore, composition and content of organic acids play an important role during the

fermentation process.

Organic acid contents in different food matrices have been analyzed using various methods, including high-performance liquid chromatography-ultra violet (HPLC-UV) detection in juices (5), solid-phase extraction followed by HPLC in liquors (6), capillary electrophoresis in wines (7), Fourier transform infrared (FT-IR) detection in wines (8), ion chromatography in liquors (9), and proton nuclear magnetic resonance (¹H-NMR) in beers (10).

Although analysis of organic acids has been frequently attempted, quantitative analysis of organic acids, especially those in liquors, has been a difficult task when easily accessible HPLC is used. Until now, studies on HPLC analysis for organic acid contents in liquors have been limited. In this study, we compared three different HPLC analysis methods for the quantitative analysis of organic acids in red wine, white wine, and beers.

Materials and Methods

Materials and reagents Seven types of standard materials (oxalic, tartaric, malic, lactic, acetic, citric, and succinic acid) were purchased from Sigma-Aldrich (St Louis, MO, USA). Monopotassium phosphate (KH₂PO₄), perchloric acid (HClO₄), bromothymol blue (BTB), sodium hydroxide (NaOH), and sodium phosphate dibasic (Na₂HPO₄) were

also purchased from Sigma-Aldrich. The samples of red wine (one foreign brand), white wine (two foreign brands), and beer (three foreign brands and two domestic brands) were purchased from a local market (Seoul, Korea). HPLC-grade solvent was used for the preparation of the mobile phase.

Pre-treatment of the samples In the case of beer possessing gas, CO₂ was removed by repeated pipetting and vortex mixer until no bubbles were generated. Beers comprising floating matters were centrifuged (Hanil HA-1000-3; Hanil Science Co., Daejeon, Korea) at 1,821*xg* for 10 min. The supernatant obtained after centrifugation and the filtrate from the samples that were filtered using a 0.45 µm Nylon filter (Waterman, Clifton, NJ, USA) were used for the analysis. For UV-detection at 210 nm, the dilution ratio was set according to the characteristics of each sample. The wines were diluted five times, whereas the beers were diluted five-to-seven times. The samples, which were analyzed using visible-detection at 440 nm, were not diluted.

Analysis methods For the UV-detection analysis at 210 nm (method 1), the analysis procedure stated in The International Organization of Vine and Wine (OIV) (11) was slightly modified and the HPLC conditions (method 1) used are presented in Table 1. The pre-treated samples were analyzed using HPLC (Younglin SP930D; Younglin, Anyang, Korea) at 210 nm. Each sample (20 µL) was injected into the HPLC system. KH₂PO₄ (50 mM dissolved in water) solution was used as the mobile phase after adjusting the pH to 2.8. The flow rate of the mobile phase was maintained at 0.5 mL/min. The HPLC analysis columns used were Waters Nova-Pak RP-C₁₈ (150 mm×3.9 mm I.D.) with a particle size of 4 µm connected to the Hypersil GOLD aQ analytical column (250 mm×4.6 mm I.D., particle size 5 µm). This method was termed as method 1.

The method using the post-column system was adopted from that stated in the application guide of Shodex (12) and Tabaru *et al.* (13) with minor modifications. The HPLC conditions (method 2 and 3) are presented in Table 1. The analysis was conducted using HPLC (Younglin 9000; Younglin), and the sample injection value was set at 20 µL. As the mobile phase, HClO₄ (3 mM) dissolved in water was

used at a flow rate of 0.9 mL/min throughout the column. The prepared BTB solution at 0.9 mL/min was used for the post-column reaction before the analysis using a visible-detector at 440 nm. A second solvent BTB solution was prepared by adding 0.2 mM BTB, 15 mM Na₂HPO₄, and 2 mM NaOH into a volumetric flask and the volume was adjusted to 1 L. SUPLECOGEL C-610H (30 cm×7.8 mm I.D., particle size 9 µm), on which the Hypersil GOLD aQ analytical column (250 mm×4.6 mm I.D., particle size 5 µm) was connected, was maintained at 80°C in the column oven. This analysis method was termed as method 2. Although the solvent used was the same, the analysis method using only one column, i.e., SUPLECOGEL C-610H, at 80°C was designated as method 3. Each sample was analyzed twice and the average measurement value was determined.

Measurement of linearity and precision Calibration curves obtained using method 1, by diluting a total of seven organic acids with the mobile phase (50 mM KH₂PO₄ in water), are shown in Table 2. During the standard preparation of oxalic acid, the concentrations were adjusted to 0.0025, 0.0125, 0.025, 0.25, and 0.5 mg/mL. The other six standards were adjusted to concentrations of 0.005, 0.025, 0.05, 0.25, 0.5, and 1 mg/mL. The calibration curves were obtained using the area and concentration of the standard solutions, and the organic acids were then quantified to obtain the relative standard deviation.

For methods 2 and 3, seven types of organic acids in the form of a mixture were prepared and the calibration curves were then obtained (Table 2). Oxalic, tartaric, and citric acids were prepared at concentrations of 0.1, 0.2, 0.3, 0.6, 0.9, 1.8, and 2.4 mg/mL. Malic, succinic, and acetic acids were prepared at concentrations of 0.075, 0.15, 0.225, 0.45, 0.675, 1.35, and 1.8 mg/mL. Lastly, the lactic acid concentrations were adjusted to 0.15, 0.3, 0.45, 0.9, 1.35, 2.7, and 3.6 mg/mL. However, acetic acid and succinic acid were not separated but co-eluted instead using method 2. Therefore, the concentrations for preparing the calibration curve of the co-eluted peaks (acetic acid and succinic acid) were determined by preparing the acids at concentrations of 0.15, 0.3, 0.45, 0.9, 1.35, 2.7, and 3.6 mg/mL. Furthermore, tartaric acid and citric acid were co-eluted using method 3. Thus, the value obtained on summing up the concentrations

Table 1. Operating conditions (Method 1, 2 and 3) for analysis of organic acids

Instrument		Method 1	Method 2	Method 3
Columns	1.	Waters Nova-Pak®RP-C ₁₈ 3.9 mm×150 mm, 4 µm particle	1. Hypersil GOLD aQ Analytical Column 250 mm×4.6 mm, 5 µm particle	1. SUPLECOGEL C-610H 30 cm×7.8 mm, 9 µm particle
	2.	Hypersil GOLD aQ Analytical Column 250 mm×4.6 mm, 5 µm particle (room temperature)	2. SUPLECOGEL C-610H 30 cm×7.8 mm, 9 µm particle (Oven 80°C)	(Oven 80°C)
Detector		UV detector (wave length=210 nm)		
Mobile Pump 1 phase	Pump 1	50 mM KH ₂ PO ₄ (dissolved in water)		
	Pump 2	No use		
Flow rate		0.5 mL/min (eluent)		
Injection volume		20 µL		
Total running time		60 min		
			30 min	20 min

Table 2. Retention times, linear range, and area percentage of organic acids

Method	Organic acids	RT (min)	Concentration range (mg/mL)	Calibration	Correlation coefficient (R^2)	Area (%)
1	Oxalic	8.1	0.0025–0.5	$y=17953x+18.823$	1	52.4
	Tartaric	8.8	0.005–1	$y=2785x+23.414$	0.9999	17.0
	Malic	10.6	0.005–1	$y=1234.6x+2.3073$	1	7.2
	Lactic	12.4	0.005–1	$y=993.95x+0.8296$	1	5.6
	Acetic	13.5	0.005–1	$y=738.27x+0.7496$	1	4.2
	Citric	15.9	0.005–1	$y=1632.6x+1.7793$	1	9.4
	Succinic	20.5	0.005–1	$y=753.83x+2.9851$	0.9998	4.3
2	Oxalic	8.3	0.1–2.4	$y=3183.8x+100.97$	0.9997	19.6
	Tartaric	9.8	0.1–2.4	$y=2143.2x+22.88$	0.9999	13.5
	Malic	11.2	0.075–1.8	$y=2246.7x+2.9361$	0.9999	11.2
	Citric	12.1	0.1–2.4	$y=2307.4x+20.931$	0.9997	16.3
	Lactic	13.8	0.15–3.6	$y=1651.5x+33.895$	0.9994	15.6
	Acetic+Succinic	15.7	0.15–3.6	$y=2400.4x+39.147$	0.999	23.8
3	Oxalic	5.0	0.1–2.4	$y=3492.8x+78.642$	1	21.4
	Tartaric+Citric	6.7	0.2–4.8	$y=2521.6x+41.531$	0.9988	23.9
	Malic	7.1	0.075–1.8	$y=2508.6x+37.34$	0.9997	10.2
	Succinic	8.7	0.075–1.8	$y=3951.2x+79.039$	0.9986	17.6
	Lactic	9.2	0.15–3.6	$y=1720x+47.731$	0.9987	15.8
	Acetic	10.9	0.075–1.8	$y=2890.5x+74.671$	0.9988	11.2

(0.2–4.8 mg/mL) of the tartaric acid and citric acid was adopted. The concentration ranges (0.075–3.6 mg/mL) of each organic acid are presented in Table 2.

Monitoring of the organic acid contents in the liquors Organic acids were analyzed using the above three methods for one red wine (foreign brand), two white wines (foreign brands), three foreign beers (one ale beer, two lager beers), and two domestic beers (one dark beer and one ale beer).

Results and Discussion

Linearity and separation sequence The linearity of the organic acid contents, obtained using the three proposed analysis methods, was studied. The calibration curve for each standard material was prepared and the R^2 (coefficient of correlation) value was determined (Table 2). The R^2 values for all the seven standard materials analyzed using methods 1, 2, and 3 were found to be higher than 0.998. The sequence of the separation with method 1 was in the order of oxalic, tartaric, malic, lactic, acetic, citric, and succinic acid (Fig. 1). The elution trends were consistent with those observed in the previous results (5). However, the analysis results using method 1 showed that the oxalic and tartaric acids were not completely separated but overlapped (Fig. 1). Moreover, though the concentration of tartaric acid was two times higher than that of oxalic acid, the oxalic response was quite prominent. The separation sequence using method 2 was in the following order: oxalic, tartaric, malic, citric, and lactic acid, with co-elution of acetic and succinic acids (Fig. 1). Using

method 3, the sequence of separation was as follows: oxalic acid, co-eluted tartaric and citric acid, followed by a sequential separation of malic, succinic, lactic, and acetic acids. At this time, the succinic and lactic acid were not separated completely (Fig. 1). Therefore, the acetic and succinic acids were co-eluted using method 2, whereas the tartaric and citric acids were co-eluted using method 3.

Monitoring the organic acid contents in the liquors in domestic markets The organic acid contents for the one red wine (RW, foreign brand), two white wines (WW, foreign brands), and five beers [two domestic brands (DB) and three foreign brands (FB)] analyzed using the above methods are provided in Table 1. The chromatograms of all the samples analyzed using method 1 exhibited an unstable baseline and reduced separation because of the presence of interfering materials. The instability and low separation could not be improved even by adjusting the concentration of the samples. Therefore, peak designation and integration for the quantitative analysis of each organic acid was not satisfied using method 1. Though the separation and detection method for the wines provided by OIV were slightly modified, the baseline of the beers was especially unstable. This might be because of the presence of various interfering materials that negatively interfere in the analysis at 210 nm using method 1. Furthermore, ethanol, present in the liquors, with a UV cut-off value at 210 nm may also have negatively affected the analysis (Fig. 2). The seven organic acid standards were separated using all the analysis methods; however, since oxalic acid was co-eluted with other materials, a quantitative analysis was not conducted.

When the wine samples were analyzed using method 1, the

Fig. 1. Chromatograms of a standard mixture of organic acids separated using three methods. (A) Method 1. 1, oxalic (0.125 mg/mL); 2, tartaric (0.025 mg/mL); 3, malic (0.025 mg/mL); 4, lactic (0.025 mg/mL); 5, acetic (0.025 mg/mL); 6, citric (0.025 mg/mL); and 7, succinic (0.025 mg/mL). (B) Method 2. 1, oxalic (0.6 mg/mL); 2, tartaric (0.6 mg/mL); 3, malic (0.45 mg/mL); 4, citric (0.6 mg/mL); 5, lactic (0.9 mg/mL); and 6, acetic+succinic 0.9 mg/mL). (C) Method 3. 1, oxalic (0.6 mg/mL); 2, tartaric+citric (1.2 mg/mL); 3, malic (0.45 mg/mL); 4, succinic (0.45 mg/mL); 5, lactic (0.9 mg/mL); and 6, acetic (0.45 mg/mL).

separation of the peaks required to determine the quantitative values of the major organic acids was not satisfactory. However, on implementing method 2, stable baselines and peaks could be obtained compared with those obtained using method 1 (Fig. 2). The analyzed red wine (RW) contained 2,652.4 mg/L tartaric acid and 1,392.9 mg/L lactic acid but relatively lower amount of malic acid (271.0 mg/L) in Table 3. This may be because of the conversion of malic acid into lactic acid through malo-lactic fermentation by lactic acid bacteria (2,3). High quality wines are generally believed to possess relatively larger amounts of lactic acid than malic acid. Meanwhile, acetic and succinic acids were co-eluted using method 2. The sum of these organic acids in the RW was 1,283.4 mg/L, whereas citric acid was not detected (Table 3). Peres *et al.* (7) investigated the contents of organic acids in Brazilian wines using capillary electrophoresis and reported that the red wines contained 1,023–2,212 mg/L tartaric acid and 35–7,306 mg/L lactic acid. Two types of red wines out of the eleven red wines tested contained approximately 2,200 mg/L of malic acid. In addition, the contents of lactic acid in the two red wines were the least at 35 and 84 mg/L among the 11

samples. Citric acid was detected in only one sample, acetic acid content was in the range of 118–1003 mg/L, and succinic acid was also present in a range of 66–700 mg/L (7). Castineira *et al.* (14) have reported that the average contents of tartaric acid, acetic acid, and succinic acid in 21 Spanish red wines were 1,195, 373, and 485 mg/L, respectively. Notably, the average content of lactic acid was 1,731 mg/L.

The content of tartaric acid in the white wine (WW-1) was 2,749.1 mg/L. Malic acid was present in a relatively lower concentration (470.2 mg/L) compared with lactic acid (1,018.5 mg/L). However, in the WW-2 sample, the concentration of malic acid (3,107.9 mg/L) was higher than that of tartaric acid (1,160.8 mg/L), whereas lactic acid was not detected (Table 2). In the results of previous studies (7), the least lactic acid content among the eight white wines was determined as 61 mg/L, whereas the malic acid content was quite high (1,996 mg/L). Citric acid, however, was not detected in the two samples (WW-1 and WW-2), consistent with the results reported by Peres *et al.* (7), who could not detect citric acid in all the eight white wines.

When the beers were analyzed using method 2, the baseline and separation of the organic acids improved greatly (Fig. 3). However, the stability of the baselines and separation of the peaks needed further refinement, thus necessitating an analysis using method 3. Though citric and tartaric acid were co-eluted using method 3, malic, lactic, acetic, and succinic acid could be fully separated (Fig. 3). The contents of the organic acids analyzed using method 3 are presented in Table 3. Malic acid contents for the three types of foreign beers (FB-1, FB-2, and FB-3) were in a range of 62.2–110.5 mg/L along with acetic acid in a range of 93.5–183.8 mg/L. The succinic acid content was found to be the lowest among the analyzed organic acids in a range of 37.0–56.2 mg/L. Lactic acid was present in a range of 95.9–226.4 mg/L. Ale beer FB-1, meanwhile, showed relatively higher contents of organic acids than the lager beers FB-2 and FB-3.

Two types of domestic beers (DB-1 and DB-2) were analyzed using method 3 (Fig. 3) and the results are presented in Table 3. The contents of malic acid in DB-1 and DB-2 were 119.2 mg/L and 97.8 mg/L, respectively. Moreover, the acetic acid contents of DB-1 and DB-2 were significantly different at 64.5 and 224.9 mg/L, respectively. Similar to the foreign beers, the contents of succinic acid in DB-1 and DB-2 were the lowest among the analyzed organic acids at 59.4 and 66.9 mg/L, respectively, whereas the lactic acid concentrations were 111.3 mg/L and 205.4 mg/L, respectively. The ale beer DB-2 showed 1.8–3.5 times higher concentrations of lactic and acetic acid than the black beer DB-1. Our results of organic acid contents in beer are fairly consistent with those of the previous reports (10,15). Nord *et al.* (10) investigated the organic acid contents for 58 types of lager beers using $^1\text{H-NMR}$. The approximate maximum values of organic acids were as follows: lactic acid, 300 mg/L; citric acid, 200 mg/L; acetic acid and succinic acid, 150 mg/L; and malic acid, 100 mg/L. Montanari *et al.* (15) separated the organic acids from four types of Italian lager beers using HPLC on which a C18 column was attached. After

Fig. 2. Chromatograms of wine samples in different methods. (A) RW-1 (method 1). (B) RW-1 (method 2; 2, tartaric; 3, malic; 5, lactic; 6, acetic+succinic). (C) WW-1 (method 1). (D) WW-1 (method 2; 2, tartaric; 3, malic; 5, lactic; 6, acetic+succinic). (E) WW-2 (method 1). (F) WW-2 (method 2; 2, tartaric; 3, malic; 6, acetic+succinic). Abbreviations as given in Table 3.

Table 3. Organic acid contents in the selected wine and beer samples (mg/L)

		Method	Tartaric	Malic	Lactic	Citric	Acetic	Succinic	Acetic+Succinic
Red wine	RW-1 ¹⁾	2	2652.4±22.4	271.0±6.6	1392.9±3.2	ND			1283.4±7.3
White wine	WW-1	2	2749.1±13.7	470.2±0.9	1018.5±9.3	ND			278.9±0.3
	WW-2	2	1160.8±20.6	3107.9±13.5	ND	ND			180.4±5.3
Foreign beer	FB-1 (ale)	3		110.5±6.4	226.4±2.5		183.8±7.2	56.2±3.1	
	FB-2 (lager)	3		62.2±6.8	188.9±21.0		128.6±0.2	37.0±1.7	
	FB-3 (lager)	3		107.0±1.3	95.9±0.9		93.5±4.2	55.3±3.8	
Domestic beer	DB-1 (dark)	3		119.2±0.3	111.3±1.6		64.5±1.9	59.4±3.2	
	DB-2 (ale)	3		97.8±12.9	205.4±9.3		224.9±6.5	66.9±14.5	

¹⁾RW, red wine; WW, white wine; FB, Foreign beer; DB, Domestic beer

treating the samples with a refractive index detector and enzymes, quantitative analysis was performed using absorption mechanism. The organic acid contents reported were as follows: malic acid, 40–93 mg/L; lactic acid, 66–186 mg/L; acetic acid, 28–159 mg/L; citric acid, 99–124 mg/L, and succinic acid, 43–116 mg/L.

Method 2 and 3 were proposed on the basis of the post-column reaction methods suggested by Shodex (12) and the previous

literature (13). Stable baselines could be maintained using these methods compared with those using the UV-detection method, when organic acids were separated in the wines and beers but detection was possible only with relatively high organic acid concentrations. Therefore, it is necessary to increase the injection volume if the sample has relatively low organic acid contents such as that in DB-2. Furthermore, separation of the co-eluted acetic and

succinic acid using method 2 and that of the tartaric and citric acid using method 3 is expected with several columns connected. On the basis of the above findings, we are planning to improve the proposed methods for the analysis of the organic acids in domestically available liquors such as *takju* and *yakju*.

In conclusion, quantification of organic acids in wines using HPLC with UV detection at 210 nm (method 1) has been limited because of unstable baselines and reduced separation. Meanwhile, using method 2 with VIS detection at 440 nm after the post-column reaction, stable baselines and peak separation were obtained for a quantitative analysis of the organic acids in spite of the co-eluted acetic and succinic acid. In beers, however, stability of the baselines and separation of the peaks needed further refinement when method 2 was used. Thus method 3 was applied, in which the citric and tartaric acid were co-eluted. Therefore, owing to the various components and their contents in different types of liquor such as wine and beer, the HPLC method for the quantitative analysis of organic acids should be customized depending on the type of the liquor.

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