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ORIGINAL PAPER

Determination of organic acids evolution during apple cider fermentation using an improved HPLC analysis method

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Abstract An efficient method for analyzing ten organic acids in food, namely citric, pyruvic, malic, lactic, succinic, formic, acetic, adipic, propionic and butyric acids, using HPLC was developed. Boric acid was added into the mobile phase to separate lactic and succinic acids, and a post-column buffer solution [5 mmol/L *p*-toluensulfonic acid (*p*-TSA) + 20 mmol/L bis (2-hydroxyethyl) iminotris (hydroxymethyl) methane (bis–tris) + 100 μmol/L sodium ethylenediaminetetraacetic (EDTA-2Na)] was used to improve the sensitivity of detection. The average spiked recoveries for the ten organic acids ranged from 82.9 to 127.9% with relative standard deviations of 1.44–4.71%. The linear ranges of determination were from 15 to 1,000 mg/L with correlation coefficients of 0.9995–0.9999. The metabolism of organic acids in cider, and the effect of nutrients including diammonium phosphate (DAP), thiamine, biotin, niacinamide and pantothenic acid on their metabolism, were studied using this method of analysis. We found that before cider brewing, additions of 200 mg/L DAP and 0.3 mg/L thiamine to apple juice concentrate results in a high quality cider.

Keywords Organic acids · Cider · HPLC · Metabolism · Growth factors

F. Zhou and B. Ji contributed equally to this work.

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Introduction

Carboxylic acids are important constitutes in many foods, either fresh products such as apples and apple juice, or products such as cider, resulting from fermentative processes. The content of organic acids in fruit juices influences their pH and flavor as well as their stability, nutritional aspects, acceptability and keeping quality.

Acids often impart flavors in addition to the sour taste. While the dominant flavor of organic acids is sourness, they also contribute bitterness and astringency; the proportions of these sensations vary with different acids [1].

Organic acids are quality indicators during cider brewing. The nature of the processes carried out in the brewing of cider is affected by the quantity and kind of acids present in apples. For example, pyruvic acid, an important intermediate product during the Embden–Meyerhof–Parnas (EMP) pathway, is affected significantly by sulfide dioxide, and indicates the course of fermentation [2, 3]. Malic acid is the main organic acid in apple juice, and can be assimilated by some yeast, resulting in its decrease varying from 5 to 40% [5]. However, its level is reduced mainly by lactic acid bacteria during the secondary bacterial fermentation (malolactic fermentation), and should achieve the desired degree of malolactic fermentation, to make a product with proper acidity, taste and stability. Compared to the decrease of malic acid in malolactic fermentation, the presence of lactic acid in cider indicates that malolactic fermentation has occurred, conferring microbial stability to the cider and resulting in a less marked sour taste [4]. Citric acid is metabolized to acetic acid, whereas shikimic and quinic acids are transformed into single phenols (catechol, ethylcatechol) and other compounds [5].

Organic acids, including tartaric acid, malic acid, citric acid, and succinic acid have characteristic buffering capacities,

which affect the yeast metabolism [6]. In addition, the inhibitory effect on microorganism of organic acids has been widely reported to be caused by their undissociated form [7, 8]. Finally, the enzymatic activity and the chemical alterations are also influenced by the acidity.

Therefore, it is important to accurately determine the organic acids for purposes of quality control, to meet the specifications, regulations, and labeling. Because of their importance, considerable effort has been made to quantify organic acids in apples and their products.

At present, organic acids are measured using enzymatic methods or liquid chromatography, ion chromatography, while some volatile organic acids such as acetic, propionic, and butyric acids are measured by GC [4, 9–14]. Enzymatic analyses, however, require specific kits for each individual organic acid; they are rather time-consuming and costly. The traditional HPLC techniques with refraction index or UV detection do not always allow the separation of minor organic acids and often require previous purification techniques to eliminate, for example, the interference of sugars or phenolic compounds. All of these have a negative influence on the simplicity and rapidity of the method, which is especially inconvenient in the case of routine quality control analysis. The introduction of conductivity detectors combined with ion chromatography with chemical suppression has eliminated many of the above-mentioned problems [15], but needs specific equipment, which is rather expensive. Recently we succeeded to achieve good separation of organic acids based on the optimization of combination of an electroconductivity detector with a traditional HPLC instrument, using a specific purification column developed by Shimadzu Inc., Japan.

The aim of the present work was to optimize the proposed method for analysis of cider samples, to measure major organic acids present in apple juice (malic and citric acids) as well as the metabolic products of fermentation (pyruvic, lactic, succinic and acetic acids) without previous purification procedures. On this basis, we studied the production and consumption of organic acids during cider brewing, and the effect of selected nutrients including diammonium phosphate (DAP), thiamine, biotin, niacinamide and pantothenic acid, on their metabolism.

Materials and methods

Instrumentation

The experiments were performed on a Shimadzu LC-10A System (Shimadzu, Tokyo, Japan), consisting of two LC-10Avp pumps, Column Oven CTO-10Avp and a Model 10Avp conductivity detector. The following columns and accessories were also from Shimadzu: Shim-pack SCR-102H (8.0 mm i.d. × 300 mm) for separation, and SCR-102H as a guard column. A T-connector was used at the outlet of the column for the incorporation of the post-column buffer.

HPLC initial analysis condition

Both mobile phase and buffer solution flow rate is 1.0 mL/min. The separation temperature is 45 °C with the conductivity detector temperature 48 °C. The injected amount is 10 μL. The result is qualitatively analyzed by peak retention time, and quantified by peak area using external standard method.

Chemicals

The mobile phase consisted of 5 mmol/L *p*-toluensulfonic acid (*p*-TSA, Aldrich, USA) and 0.4 mol/L boric acid (Sigma, USA) in redistilled water. The buffer solution (pH 7.04) consisted of *p*-TSA (5 mmol/L), 20 mmol/L bis (2-hydroxyethyl) iminotris (hydroxymethyl) methane (bis–tris, Sigma, USA), and 100 μmol/L sodium ethylenediaminetetraacetic (EDTA-2Na, Wako Pure Chemical Inc., Japan) in redistilled water.

Calibration curves were prepared using acid solutions in redistilled water prepared with citric, pyruvic, malic, succinic, formic (approximately 99%), acetic, adipic, butyric (98%) (Wako, Japan, provided by Shimadzu Corp., Japan), lactic (88.4%) and propionic acids (99.5%) (Sigma-Aldrich, USA).

Diammonium phosphate (DAP), thiamine, biotin, niacinamide and pantothenic acid (Amresco, USA) were added as the nutrients during the study of evolution of organic acids content.

Standard solutions

For all the acids, stock solutions of 1,000 mg/L were prepared monthly in redistilled water, and standard solutions of suitable concentration were made to obtain calibration curves over a linear range, depending on the levels that naturally occur in apples and cider. Quantification was based on peak area measurements.

Sample preparation

All the samples were diluted with redistilled water to obtain concentrations of organic acids within the linear range of the calibration curves, then filtered through 0.45 μm filters and frozen at −18 °C. No further pre-treatment of samples, such as elimination of sugars and phenols were required.

Fermentation experiments

The control fermentation was carried out as follows. Apple juice concentrate was diluted to 23°brix, (corresponding to a total sugar concentration of approximately 200 g/L), 80 mg/L SO_2 was added, and was inoculated at a level of 5% (v/v) with *Saccharomyces cerevisiae* J00 (precultivated at 25 °C for 48 h at 150 rpm), and fermented at 18 ± 1 °C for 18 days protected from light. During fermentation, samples were taken at 48 h intervals for the determination of organic acids.

The effects of the nutrients DAP, thiamine, biotin, niacinamide and pantothenic acid, which added to the diluted apple juice before inoculation, was tested in single factor experiments (Table 1).

Results and discussion

Optimization of the separation

Evaluations were carried out with a test mixture consisting of citric, tartaric, pyruvic, malic, succinic, lactic, formic, acetic, adipic, propionic and butyric acids. The initial mobile phase was a solution of 5 mmol/L *p*-TSA. This could neither adequately separate citric and pyruvic acids, nor lactic and succinic acids, thus modifications were developed as described below.

Use of a post-column buffer solution

Since the use of acidic water as the mobile phase increases the electroconductivity relative to the background value and

Table 1 Effect of growth factors on organic acid metabolism etc

Growth factor	Level	Added (mg/L)	Pyruvic acid (mg/L)	Malic acid (mg/L)	Lactic acid (mg/L)	Succinic acid (mg/L)	Acetic acid (mg/L)	Citric acid (mg/L)
Control	0	0	610.83	5783.23	195.25	213.07	84.02	695.35
DAP			***	**	**	**	**	***
	1	200	544.815	5565.05	256.84	264.5145	72.05	768.60
	2	400	590.07	5725.53	291.31	268.855	60.04	847.35
	3	600	637.02	4465.45	216.04	213.494	91.13	899.99
	4	800	703.98	5963.63	221.24	231.907	58.18	1034.99
	5	1,000	725.09	5806.80	215.84	232.5357	77.89	1144.14
Thiamin			***	**	***	***	**	***
	1	0.3	433.74	5474.5	121.96	416.92	76.61	610.22
	2	0.6	421.66	5591.5	115.09	432.17	65.13	594.70
	3	0.9	399.01	5499.4	121.93	373.38	70.95	536.37
	4	1.2	426.98	5375.55	103.12	387.58	105.52	576.04
	5	1.5	420.73	5783.8	117.99	384.80	68.85	584.69
Biotin	1	0.1	603.85	6004.45	193.14	191.04	105.05	729.75
	2	0.2	587.83	5635.05	222.23	219.46	58.03	757.73
	3	0.3	540.32	6046.65	160.46	184.05	91.01	694.83
	4	0.4	613.59	6176.35	261.29	278.62	77.84	857.82
	5	0.5	534.31	5559.90	177.60	183.86	77.78	679.53
Niacinamide			**	***			**	
	1	5	711.25	5372.35	230.37	268.39	271.69	765.77
	2	10	685.93	4648.90	237.56	225.34	237.52	732.23
	3	15	662.85	4912.00	251.22	263.07	207.72	717.82
	4	20	712.61	5355.35	224.83	268.27	295.45	772.34
	5	25	737.89	5423.10	292.86	286.89	215.89	774.14
Panthotenic acid	1	1	529.82	5746.15	222.18	208.29	50.49	724.94
	2	2	580.62	5734.80	214.13	224.65	117.25	774.87
	3	3	598.77	5340.30	197.20	189.53	90.81	737.49
	4	4	512.74	5295.05	192.19	184.87	61.79	685.30
	5	5	493.24	5344.475	189.86	186.75	61.12	657.43

* Significant at $P < 0.1$, ** significant at $P < 0.05$, *** significant at $P < 0.001$

decreases response to object organic acids due to suppression of their dissociation, a post-column pH-buffer could reduce the electroconductivity of the background and facilitate the dissociation of the organic acids, permitting the combination of ion exclusion chromatography and electroconductivity detection. To protect the column, chelated reagent EDTA is added to let the ions complexed with EDTA, but not with the chromatography column. Except for acids of low pKa value, such as pyruvic acid the detection of most acids was improved. The best results were obtained with a buffer solution (pH 7.04) containing *p*-TSA (5 mmol/L), bis–tris (20 mmol/L) and EDTA (100 μmol/L). EDTA may be replaced by the more easily soluble EDTA-2Na, although this may cause a slight increase of baseline noise.

Concentration of the *p*-TSA in the mobile phase

The concentration of *p*-TSA in the buffer solution is the same as in the mobile phase. Higher concentrations of *p*-TSA cause increased pH values of the mobile phase. In general, lower pH results in less dissociation of the organic acids and thus in better separation on ion exclusion columns. Citric and pyruvic acids separated well at *p*-TSA concentrations (5 mmol/L, whereas the separation of pyruvic and malic acids was jeopardized at *p*-TSA concentrations of $\geq$10 mmol/L. So for citric and pyruvic acids, the appropriate concentration was 5 mmol/L. However, the fermentation products (lactic and succinic acids) could not be separated successfully with this mobile phase.

Composition of the mobile phase

Boric acid was added to the mobile phase to improve the separation of lactic and succinic acids, based on the assumption that reactions between α-hydroxy of lactic acid and boric acid would improve the separation [16]. We found that a combination of 5 mmol/L *p*-TSA and 0.4 mol/L boric acid was the best mobile phase for the separation of lactic and succinic acids, while this did not affect the separation of other acids.

Separation temperature

The temperature of the column is an important dynamic factor influencing the extent of separation and column efficiency. Higher column temperatures result in higher dissociation rates of weak organic acids, and shorter retention times. So, high column temperatures are neither favorable for the separation of the acids, nor for the lifetime of the column. Nevertheless, we observed that the separation of succinic acid from lactic acid was improved at slightly elevated temperatures. Restricted by the range of the detector cell, three column temperatures: 35, 40 and 45 °C were studied with other conditions fixed, and finally 45 °C was chosen as the best separation temperature.

Flow-rate of the mobile phase

The flow-rate of the mobile phase is another dynamic factor for the degree of separation and column efficiency. We observed that the extent of separation was only slightly influenced within the range of 0.3, 0.5, 0.8, 1.0 mL/min. Considering the retention time, column pressure and analysis time, 0.8 mL/min was chosen as the final separation condition, resulting in all of the organic acids being separated within 20 min.

As a result, the combination of optimum conditions consisted of the use of an ion exclusion column (8.0 mm i.d. × 300 mm), with a mobile phase of a mixture of *p*-TSA (5 mmol/L) and boric acid (0.4 mol/L) at a temperature of 45 °C and a flow rate of 0.8 mL/min. The buffer solution (pH 7.04) consisted of *p*-TSA (5 mmol/L), bis–tris (20 mmol/L) and EDTA-2Na (100 μmol/L). Detected by electroconductivity detector, the ten organic acids were separated well, as shown in Fig. 1a.

Table 2 presents calibration data for ten organic acids tested with seven replications. Regression equations were obtained from eight concentrations of acid standards varying from 10 to 1,000 mg/L. The standard deviations of the slopes are given, as well as the correlation coefficients (R^2), which indicate a very good fit of the straight line. Linear ranges for all acids reach up to 1,000 mg/L in a uniform manner, enabling simultaneous quantification of all the acids. The limit of the detection (LOD) was calculated at a signal/noise (S/N) ratio 3, and range from 4.5 to 22.3 mg/L which is adequate for the study of fermentation processes. Relative standard deviation (RSD) of all the organic acids' value was range from 1.44 to 4.71%, which was acceptable for our analytical purpose. Recovery was calculated at acid concentrations of 50, 100, 200 and 500 mg/L, in the presence of 10% alcohol, which is similar to the concentration in cider. Recoveries were adequate. We conclude that this method has a high selectivity and reproducibility, and that its baseline does not drift, in contrast to ion chromatography and the traditional UV detection method [14]; this is helpful in the accurate and simultaneous quantification of organic acids.

Study on evolution of organic acids content

In order to test the method on food products we compared concentrated apple juice (Fig. 1b) with 18 days fermented cider (Fig. 1c). Figure 1b, c indicate that pyruvic acid was produced during fermentation; its maximum production was reached when half of the sugar was consumed (data not shown). Lactic, succinic and acetic acids are also produced during fermentation; their levels in initial apple juice are

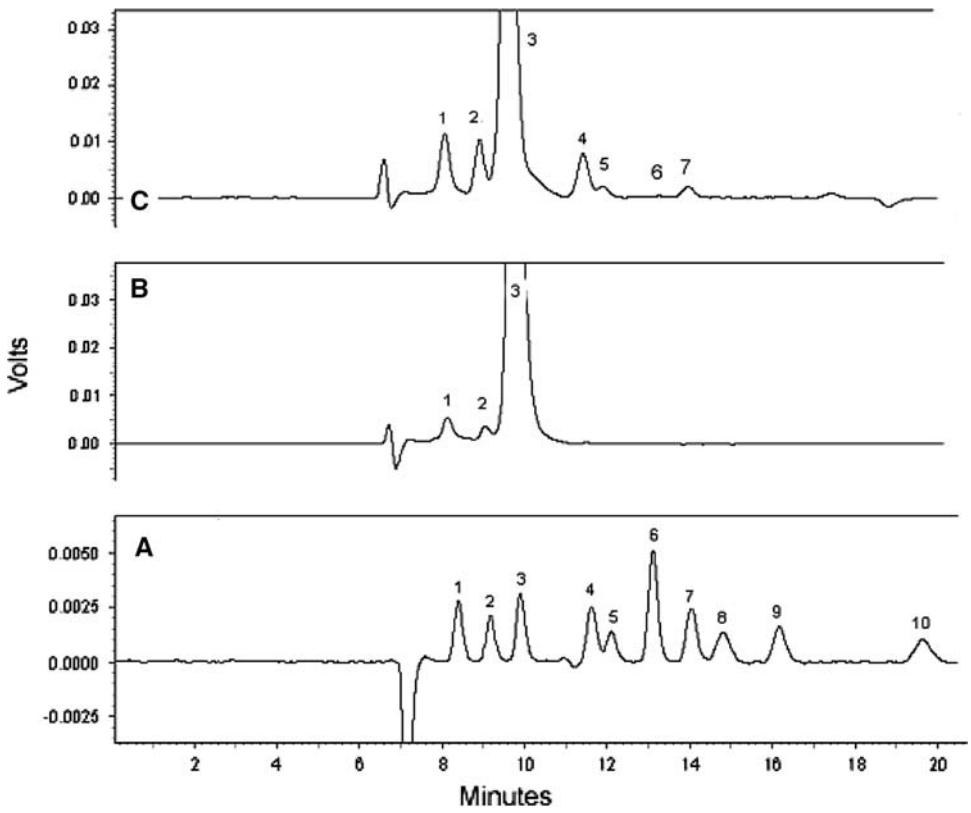


Fig. 1 **a** Chromatogram of a mixture of ten organic acids standards. **b** Organic acids in cider at the start of the alcoholic fermentation. **c** Organic acids in cider after 18 days of alcoholic fermentation

Table 2 Calibration curves and linear ranges of ten organic acids

	Organic acid	Regression equation	R^2	*P* value		RSD (%)	Linear range (mg/L)	LOD (mg/L)	Recovery (%)
				Variable of *x*	Intercept				
1	Citric acid	$y = 3.73 \times 10^2x + 2.84 \times 10^3$	0.9999	0.001***	0.031**	1.47	29.2–1,000	8.76	94.4–100.0
2	Pyruvic acid	$y = 2.54 \times 10^2x + 1.89 \times 10^3$	0.9997	0.001***	0.080*	1.89	40.3–1,000	12.1	95.3–103.2
3	Malic acid	$y = 4.48 \times 10^2x + 0.934 \times 10^3$	0.9999	0.001***	0.360	1.44	25.6–1,000	7.68	97.2–100.0
4	Succinic acid	$y = 2.11 \times 10^2x + 4.81 \times 10^3$	0.9996	0.001***	0.097*	3.97	59.7–1,000	17.9	82.9–127.9
5	Lactic acid	$y = 3.76 \times 10^2x + 7.90 \times 10^3$	0.9998	0.001***	0.192	2.36	34.0–1,000	10.2	91.5–113.1
6	Formic acid	$y = 7.99 \times 10^2x + 6.20 \times 10^3$	0.9999	0.001***	0.047**	1.61	15.0–1,000	4.50	95.1–110.9
7	Acetic acid	$y = 4.33 \times 10^2x + 4.17 \times 10^3$	0.9998	0.001***	0.047**	2.59	30.5–1,000	9.15	92.1–113.3
8	Adipic acid	$y = 3.26 \times 10^2x + 3.44 \times 10^3$	0.9998	0.001***	0.048**	4.71	55.3–1,000	16.6	86.6–100.9
9	Propionic acid	$y = 3.24 \times 10^2x + 1.46 \times 10^3$	0.9999	0.001***	0.161	3.56	48.3–1,000	14.5	91.1–102.5
10	Butyric acid	$y = 2.70 \times 10^2x + 0.205 \times 10^3$	0.9995	0.001***	0.109	3.19	74.3–1,000	22.3	95.0–113.2

RSD Relative standard deviation. *y* peak area; *x* organic acid concentration (mg/L)

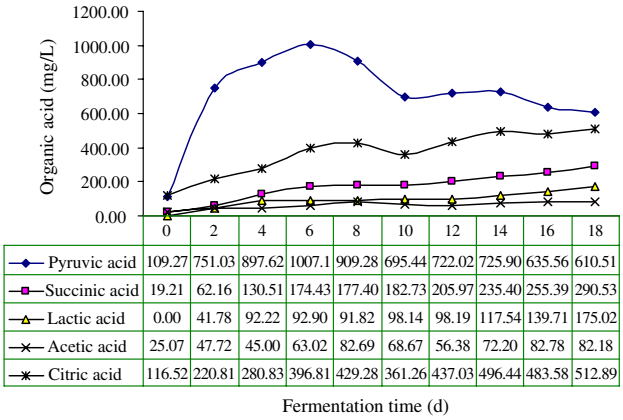
* Significance at $P < 0.1$, ** significant at $P < 0.05$, *** significant at $P < 0.001$

lower than 10 mg/L. In cider, the concentrations of these acids, for example acetic acid, were approximately 200 mg/L, a value which is similar to that reported by Whiting [5]; acetic acid is important for cider, as it is a precursor for ethyl acetate which imparts a fruity note to the beverage.

Detected by our improved method, we studied the evolution of organic acids content during cider brewing, as well as the effect of added nutrients, as reported below.

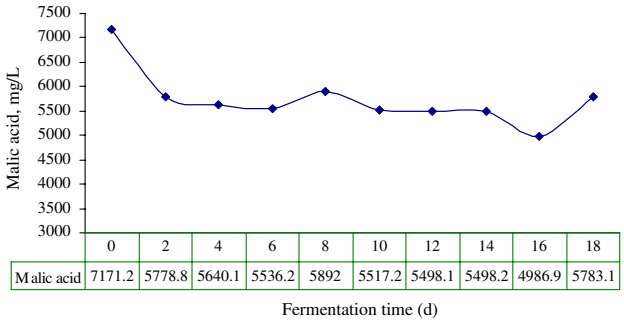
Pyruvic acid

Pyruvic acid is an intermediate in the EMP glycolysis and a precursor for many other substances. In spite of its great importance as a metabolic intermediate it is excreted by yeast during cider fermentation with SO_2 [7], sometimes at high concentrations, ranging from 80 to 640 mg/L. Figure 2 shows that pyruvic acid was excreted and its maximum



	0	2	4	6	8	10	12	14	16	18
Pyruvic acid	109.27	751.03	897.62	1007.1	909.28	695.44	722.02	725.90	635.56	610.51
Succinic acid	19.21	62.16	130.51	174.43	177.40	182.73	205.97	235.40	255.39	290.53
Lactic acid	0.00	41.78	92.22	92.90	91.82	98.14	98.19	117.54	139.71	175.02
Acetic acid	25.07	47.72	45.00	63.02	82.69	68.67	56.38	72.20	82.78	82.18
Citric acid	116.52	220.81	280.83	396.81	429.28	361.26	437.03	496.44	483.58	512.89

Fig. 2 Concentration change of citric, pyruvic, succinic, lactic, and acetic acids during alcoholic fermentation in cider



	0	2	4	6	8	10	12	14	16	18
Malic acid	7171.2	5778.8	5640.1	5536.2	5892	5517.2	5498.1	5498.2	4986.9	5783.1

Fig. 3 Concentration change of malic acid during alcoholic fermentation in cider

content occurs when approximately half of the sugar has been fermented (day 6). Subsequently, it is taken up again and metabolized further but the amounts taken up by the cells may vary considerably [5]. At the end of fermentation, the content of pyruvic acid reached 610.51 mg/L. Several factors influence the excretion of pyruvic acid, including yeast strain and medium composition. Thiamine deficiency, which usually caused by addition of SO_2 has been reported to increase the amount of excreted pyruvic acid six times, and adequate supply lowered its excretion [3, 5]. Table 1 shows that the addition of 0.3 mg/L of thiamine to apple juice can effectively decrease about 29.0% pyruvic acid in cider. Nitrogen is an important nutrient for the oxo-acid excretion, and adequate addition of single amino acids into juice containing 150–180 g/L sugar was reported to significantly increase the pyruvic acid level [5]. Our result in Table 1 shows that addition of 200 mg/L DAP could reduce excretion of pyruvic acid, but with increased addition of DAP, the excretion of pyruvic acid would increase by 18.7% (725.09 mg/L). This demonstrates that excessive DAP is detrimental to fermentation. Deficiency of either niacinamide or pantothenic acid under aerobic condition reportedly increased pyruvic acid by approximately 50% [5]. However, fermentation under anaerobic conditions has not been reported. In our study, 5 mg/L niacinamide enhanced the secretion of pyruvic acid. Biotin and pantothenic acid showed no significant effect on pyruvic acid metabolism. The one-way analysis of variation (ANOVA) (Table 1) indicated that DAP, thiamine and niacinamide significantly affect the metabolism of pyruvic acid.

Malic acid

As the major acid, high content of malic acid will cause harsh flavor of cider. Yeasts may either break down or form malic acid during fermentations. Figure 3 demonstrates that *S. cerevisiae* J00 could degrade malic acid. The varying concentration of malic acid during fermentation could result from its involvement in several biochemical pathways. Decomposition of malic acid is affected by some factors, e.g. content of vitamins and nitrogen. In cider fermentations, it was reported [5] that *S. cerevisiae* caused little change in malic acid content at pH 4.0 but at pH 3.0 an appreciable decrease (16% for Champagne yeast) occurred in a low nitrogen juice, while in high nitrogen juices an even greater decrease took place. Table 1 illustrates that addition of 600 mg/L DAP promotes malic acid degradation at pH 3.6 of cider. The addition of 10 mg/L niacinamide also facilitates the breakdown of malic acid. Addition of 1.2 mg/L thiamine diminishes malic acid as well. The ANOVA (Table 1) indicates that DAP, niacinamide and thiamine have significant effects on the decomposition of malic acid.

Lactic acid

Lactic acid is formed by reduction of pyruvic acid and transformation of malic acid. In cider fermentations of low-thiamine juice, D-lactate was formed at concentrations three times higher than in high-thiamine juices [5]. Figure 2 shows that lactic acid was formed all the time during fermentation, and its content reached 175.02 mg/L at the end of the fermentation. Table 1 illustrates that 0.3 mg/L added thiamine could reduce the level of lactic acid significantly, which confirmed earlier reports that lack of thiamine leads to the reduction of pyruvic acid to lactic acid, instead of ethanol. Additions of 400 mg/L DAP significantly promoted the yield of lactic acid.

Succinic acid

All the yeasts would excrete succinic acid during fermentation, and its concentration in the final products varies widely, from trace to 1.6 g/L in cider [5]. It was transformed from malic acid under anaerobic conditions [5]. Like in wines, succinic acid in cider was formed when the initial juice was low in acidity; pH had not much effect over

the range from 3.0 to 3.8 [5]. Figure 2 shows that 44.9% of succinic acid was formed in 4 days, but later the composition velocity was slower. Table 1 clarified that 0.3 mg/L thiamine could significantly increase the content of succinic acid at the ratio of 95.7%. Addition of 400 mg/L DAP could significantly promote the yield of succinic acid. The ANOVA showed (Table 1) that DAP and thiamine, with the addition of 400 and 0.3 mg/L, respectively have significant effect on the yield of succinic acid, and which could enrich the cider flavor.

Acetic acid

Acetic acid is always the main volatile acid in alcoholic beverages, also an important factor to the final product of cider, for it is the former of ethyl acetate, and has a stand out and stimulate flavor itself. In other words, the content of acetic acid could illustrate the resin of cider, which is an important index of cider quality. Figure 2 showed that the content of acetic acid maintained an ascending trend and reached 93.94 mg/L after alcohol fermentation. Yeast strain, medium constituents and fermentation conditions each affect acetic acid metabolism. Omitting of biotin, pantothenic acid or thiamine from medium increased acetic acid [5]. Table 1 indicated that addition of DAP with 400 mg/L or thiamine with 0.6 mg/L could obviously lower acetic acid secretion; however, the addition of biotin and pantothenic acid is of no significance to the secretion of acetic acid. A little niacinamide with 5 mg/L addition could bring great increase of acetic acid production, with the increase rate of 223%, which is compliant with previous report [5]. The ANOVA (Table 1) showed that addition of DAP, thiamine or niacinamide are all have significant effect to the secretion of acetic acid.

Citric acid

Citric acid, like malic acid, can be formed initially in fermentation by *S. cerevisiae* and later taken into the cell and catabolized [5].

During cider fermentation, there is occasionally exterior addition of citric acid to adjust the acidity of juice and complex the ions for the stability sake. Figure 2 shows that citric acid was synthesized by *S. cerevisiae* J00 under the anaerobic condition, and the content reached 512.89 mg/L. The result in Table 1 showed that more DAP added, the higher content of citric acid, when DAP was added with 1,000 mg/L, the content of citric acid increased at the rate of 67%. Contrary, thiamine could significantly decrease the content of citric acid, with the addition of 0.9 mg/L, citric acid decreased rate was 23.7%. The ANOVA (Table 1) showed that addition of DAP or thiamine are all have significant effect to the metabolism of citric acid.

According to the above results, it is recommended that before cider brewing, appropriate amount of 200 mg/L DAP, and 0.3 mg/L thiamine should be added to the apple juice concentrate, to acquire a high quality cider with low sugar residual, high content of alcohol, low accumulation of pyruvic and malic acid, high content of lactic and succinic acid and medium content of acetic acid. Though the addition of niacinamide with 10 mg/L could promote the degradation of malic acid and the yield of acetic acid, it increased the accumulation of pyruvic acid, which is disbenifit to cider production, so is not recommended.

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