Determination of Organic Acids in Honey by Liquid Chromatography with Tandem Mass Spectrometry

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Abstract
Organic acids in honeys are currently quantified by enzymatic, capillary zone electrophoresis, and chromatographic methods; however, these methods have long analysis times and low sensitivities and selectivities. Here, we developed a simple, rapid liquid chromatography–tandem mass spectrometry (LC-MS/MS)–based method for the determination of organic acids: gluconic acid, tartaric acid, malic acid, citric acid, and succinic acid in honey. First, we evaluated the linearity, matrix effects, detection limits, quantification limits, and repeatability of our method; the limit of detection for organic acids ranged from 0.005 to 0.70 mg/kg. In this study, it was 8 to 31 times higher sensitive than the high-performance liquid chromatography method. We then used our method to determine the concentrations of gluconic acid, tartaric acid, malic acid, citric acid, and succinic acid in 25 honey samples. Gluconic acid was found to account for 64.6 to 99.8% of the total organic acids in the samples. From the correlation and statistical analysis of each component, it is considered that the organic acid is produced by worker bee enzymes such as glucose oxidase and the TCA cycle. The developed method, which has high sensitivity and selectivity, will enable the analysis of more complex and low-concentration components.

Keywords LC-MS/MS · HPLC · Matrix effect · Standard addition method

Introduction

Although organic acids represent only a small proportion (< 0.5%) of the constituents of honey, they play important roles in defining a honey's color, flavor, pH, and antimicrobial and antioxidant activities (da Silva et al. 2016; Mato et al. 2006a, b). The organic acid composition of honey can also be used to

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discriminate its botanical origin (Daniele et al. 2012). Thirty non-aromatic organic acids (e.g., citric acid, malic acid, lactic acid, succinic acid) are reported to be present or probably present in honey (Mato et al. 2003). The main non-aromatic organic acid in honey is gluconic acid (Stinson et al. 1960), which is derived from two main sources: (i) the action of bee glucose oxidase on nectar glucose and (ii) the metabolic activity of certain *Gluconobacter* spp. (Mato et al. 2003). The identification and quantification of organic acids in organic materials require stable, sensitive analytical techniques. Currently, the organic acid compositions of organic materials are determined by three main methods: enzymatic methods (Mato et al. 1998a), capillary zone electrophoresis methods (Mato et al. 2006b), and chromatography methods (Cherchi et al. 1994; Lin et al. 2011). Variations of these methods have been developed to allow determination of organic acids in a wide range of foods, including wines (Mato et al. 2005), fruit juices (Lin et al. 2011; Mato et al. 2005), fruits (Sandin-Espana et al. 2016), and vegetables (Fernández-Fernández et al. 2010; Hernandez et al. 2009).

In general, the advantages of enzymatic methods are their high specificity, low cost, and simple instrumentation (Mato et al. 1998a, b). However, enzymatic methods are time-consuming, in that they require a different analytical method for

each target organic acid. Capillary zone electrophoresis is often used to examine organic acids due to its ability to be automated, short analysis times, and simple sample preparation (Castiñeira et al. 2002; Mato et al. 2006b); however, it has low sensitivity for the detection of most organic acids. Among the chromatographic methods available, gas chromatography can be used for the analysis of some organic acids. However, gas chromatography methods need a derivatization process because most organic acids are non-volatile (Horváth and Molnár-Perl 1998; Verzera et al. 2001). Moreover, the high temperatures used in gas chromatography may lead to sample decomposition. Liquid chromatography (LC) has been widely used to determine organic acids, with the separation and quantification of organic acids usually carried out by high-performance liquid chromatography (Chinnici et al. 2005; Lin et al. 2011). However, the ultraviolet and refractive index detectors used in these methods have low sensitivity and low selectivity in complex matrices.

Recently, liquid chromatography–tandem mass spectrometry (LC-MS/MS) has attracted attention for the analysis of organic acids because of its high mass selectivity and sensitivity (Flores et al. 2012; Wang et al. 2015); however, to our knowledge, LC-MS/MS has not been used to analyze the organic acid content in honey. It is known that lots of organic acids besides gluconic acid are present at low concentrations in honey; therefore, an analytical method with high sensitivity and selectivity is needed. First, the purpose of this study was to investigate matrix effects in honey by LC-MS/MS. Furthermore, detection limits, quantification limits, and repeatability using the standard addition method for organic acids (gluconic acid, tartaric acid, malic acid, citric acid, and succinic acid) in honey analysis. Finally, 25 honeys were analyzed and investigated the characteristics of each component by correlations between organic acid concentrations and statistical analysis.

Experimental Section

Reagents

The following analytical standards were purchased from Wako Pure Chemical Industries (Osaka, Japan): 50% gluconic acid solution (> 50% in water), tartaric acid (> 99.5%), DL-malic acid (> 98%), citric acid (> 98%), and succinic acid (> 99.5%). Ultrapure water was prepared with a Milli-Q system (18.2 MΩ.cm; Millipore, Bedford, MA). For the LC eluent, acetonitrile (CH_3CN, > 99.8%) and formic acid (CH_2O_2, > 98.0%) were purchased from Wako Pure Chemical Industries. For gluconic acid analysis, sodium hydroxide (NaOH) and hydrochloric acid (HCl, 35.0–37.0%) were purchased from Wako Pure Chemical Industries. To determine the linearity of the detector response, standard solutions were prepared and diluted in ultrapure water.

Honey Samples

Samples of 25 commercial and manuka honeys were purchased directly on the market (China, 3; Japan, 10; Spain, 2; Romania, 1; Canada, 2; USA, 1; Argentina, 1; New Zealand, 5) and were stored in darkness at room temperature (between 20 and 25 °C). The samples were diluted with ultrapure water 400-, 1000-, or 2000-fold (w/v) for determination of the concentration of gluconic acid and 80-fold (w/v) for determination of the concentrations of the other organic acids. For determination of the concentration of gluconic acid, the pH was adjusted to approximately 10.5 by using the necessary quantity of 1.0 M NaOH for hydrolysis of glucono-δ-lactone to gluconic acid, the mixture was stirred for 10 mins using a shaker, and then the pH was adjusted to approximately 7.8 by using the necessary quantity of 1.0 M HCl (Mato et al. 2006b). After using an ultrasonic bath for 15 min to dissolve the samples in the ultrapure water, the samples were filtered through a 0.45-μm membrane filter (Minisart RC hydrophilic 17762Q; Nippon Genetics Co., Tokyo, Japan).

Instrumentation for LC-MS/MS

The LC-MS/MS system comprised a LC system (Shimadzu Co., Kyoto, Japan) and a tandem mass spectrometry system (TSQ Quantum Mass Spectrometer System; Thermo Fisher Scientific, Waltham, MA, USA). The LC system was controlled via LCsolution (ver. 1.25; Shimadzu Co.) installed on a PC running Windows 7 Ultimate. The TSQ instrument and the data acquisition system were controlled via Xcalibur 2.0 SR2 (Thermo Fisher Scientific) installed on a PC running Microsoft Windows XP Professional.

Chromatographic separation was carried out using an InertSustain AQ-C18 analytical column (250 mm × 4.6 mm, 5-μm particle size; GL Sciences, Tokyo, Japan) at 40 °C. The eluent used for the separation consisted of formic acid (A) (0.1% (v/v) diluted by ultrapure water) and acetonitrile (B). The gradient flow rate was set at 0.4 mL/min. The total run time was 20 min. For the first 2 min, the mobile phase was 95% solution A and 5% solution B; from 2 to 2.5 min, the percentage of solution A was changed linearly to 85% solution A:15% solution B. From 2.5 to 15 mins, isocratic elution was maintained with 85% solution A:15% solution B. The injection volume was 10 μL for all samples. The most intense ion was chosen for mass detection and quantification. To check instrumental stability, the standard gluconic acid solution was analyzed after every 10 samples.

The operational parameters of the TSQ instrument were as follows: spray voltage, 4000 V; vaporizer temperature, 327 °C; sheath gas pressure, 50 psi; ion sweep gas

pressure, 2 psi; auxiliary gas pressure, 15 psi; and capillary temperature, 280 °C. Electrospray ionization negative ion mode provided the best response for all organic acids. The m/z values were $195 \rightarrow 75$ for gluconic acid, $149 \rightarrow 87$ for tartaric acid, $133 \rightarrow 115$ for malic acid, $191 \rightarrow 111$ for citric acid, and $117 \rightarrow 73$ for succinic acid. Several different collision energy and skimmer offset values from 5 to 30 V and 0 to 25 V were tested to determine the values that provided the optimal response by each organic acid. Table 1 shows the optimal conditions for the target organic acids. Figure 1 shows ion chromatograms of a standard of the five target organic acids. The measurements were conducted in triplicate.

Method Validation

We validated our LC-MS/MS approach by constructing calibration curves and evaluating linearity, matrix effects, detection limits, quantification limits, and repeatability. Solvent calibration curves were constructed by using different concentrations of the standard reagents in the range 0.5 to 20 mg/kg ($n = 5$) for gluconic acid and 0.5 to 10 mg/kg ($n = 4$) for the other organic acids. Matrix calibration curves were constructed by injecting four different concentrations (0, 0.5, 1, 5 mg/kg) of the organic acids on a non-spiked honey sample based by Commission Decision 2002/657/EC 2002. The matrix effect was evaluated by using the solvent slope to matrix slope ratio (Ss/Sm). The limits of detection (LOD) and quantification (LOQ) were determined as the values for which the signal-to-noise ratio at a spiked concentration of 0.5 mg/kg ($n = 3$) was 3 and 10, respectively, based by Flores et al. (2012). LC reproducibility was determined by using the relative standard deviation (RSD): the intra-day RSD was evaluated by comparing 10 replicate measurements of standard reagents containing 1 and 5 mg/kg organic acids within a single day; the inter-day RSD was evaluated by determining the concentration or organic acids in the standard reagents on three separate days.

Results and Discussion

Calibration Curves and Matrix Effect

The coefficient of determination (R^2) was between 0.985 and 0.999 for all of the target organic acids. The R^2 for all the calibration curves were > 0.99, so it was reasonable (Table 2).

Components in the matrix other than the target molecules can cause enhancement or suppression of the ion signal when using LC-MS/MS (Van Eeckhaut et al. 2009), which can have a marked impact on the obtained results. Ss/Sm > 1 and Ss/Sm < 1 indicate suppression and enhancement of the ion signal, respectively. When the 25 honey samples were analyzed, the Ss/Sm values of gluconic acid and tartaric acid ranged from 0.31 to 0.66 and 0.13 to 0.28, respectively, indicating that the matrix had enhancement effect on the ion signal (Supplementary Table 1). In contrast, the Ss/Sm values of malic acid, citric acid, and succinic acid ranged from 0.62 to 2.32, 0.73 to 1.09, and 0.46 to 1.68, respectively, indicating that the matrix had either a suppressive or enhancement effect on the ion signal, depending on honey tested (Supplementary Table 1). Based on these results, we used the standard addition protocol to determine the organic acid concentrations in the honey samples. For example, Supplementary Fig. 1 shows the solvent calibration curves and matrix calibration curves for the organic acids in honey sample 19. As the results, the R^2 of spiked honey samples was between 0.974 and 1.000 of the target organic acids for 25 honey samples.

Detection Limits and Quantification Limits

The LOD of our method for the 25 honey samples ranged from 0.03 to 0.43 mg/kg (arithmetic mean (average; ave.), 0.13) for gluconic acid, 0.005 to 0.09 mg/kg (ave. 0.03) for tartaric acid, 0.01 to 0.70 mg/kg (ave. 0.10) for malic acid, 0.02 to 0.24 mg/kg (ave. 0.06) for citric acid, and 0.02 to 0.24 mg/kg (ave. 0.09) for succinic acid (Table 3). The LODs of 2.0 mg/kg for gluconic acid, 1.0 mg/kg for tartaric acid, 1.0 mg/kg for malic acid, 1.0 mg/kg for citric acid, and 0.7 mg/kg for succinic acid reported using high-performance

Table 1 Retention times and LC-MS/MS conditions for the target organic acids

Sample	Retention time (min)	Precursor ion m/z [M-H]$^-$	Product ion m/z	Identity of main fragment	Collision energy [V]	Skimmer offset [V]
Gluconic acid	8.5	195	75	[M–2CO$_2$–O–OH]$^-$	20	5
Tartaric acid	8.6	149	87	[M–H–CO$_2$–H$_2$O]$^-$	15	0
Malic acid	9.7	133	115	[M–H–H$_2$O]$^-$	15	0
Citric acid	11.6	191	111	[M–H–CO$_2$–2H$_2$O]$^-$	15	5
Succinic acid	14.3	117	73	[M–H–CO$_2$]$^-$	15	5

Fig. 1 Ion chromatograms of standard reagents (10 mg/kg) of the five target organic acids
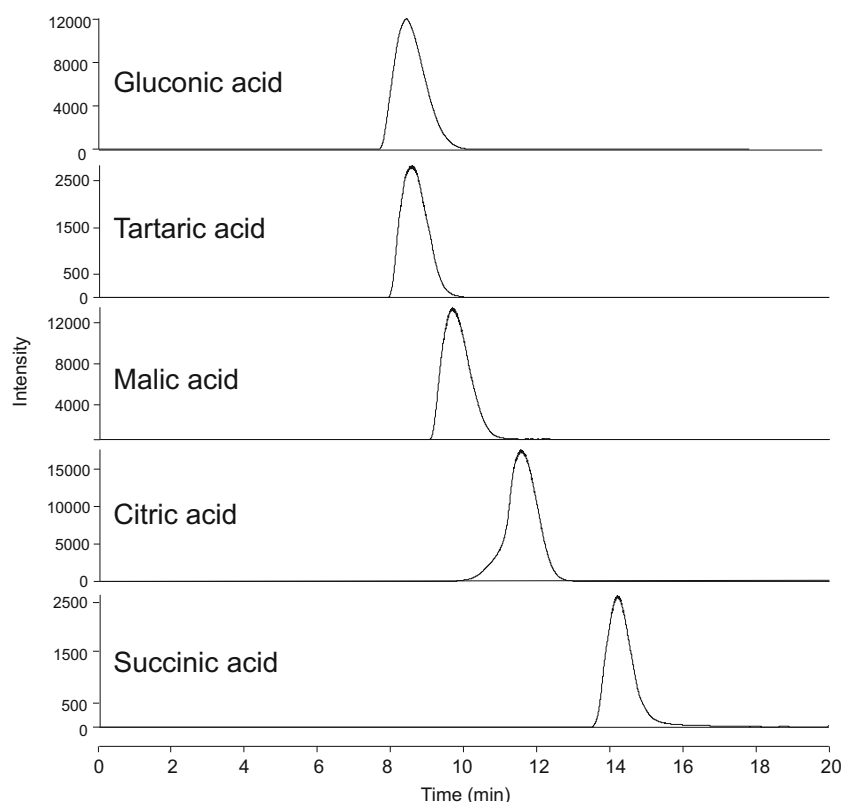
liquid chromatography (Cherchi et al. 1994) are 8.0 to 31.1 times lower sensitivity than the LODs we obtained using our LC-MS/MS method. Similarly, LODs for an enzymatic method were 0.5 mg/kg for gluconic acid, malic acid, and citric acid and 0.6 mg/kg for succinic acid (R-Biopharm enzymatic test kits with catalogue nos. 10428191035, 10139068035, 10139076035, and 10176281035, respectively), which are values 4.0 to 8.0 times lower sensitivity than the LODs we obtained using our LC-MS/MS method.

Moreover, a previous LC-MS/MS analysis of organic acids in fruits (melon, grape, peach, orange, and lemon) and vegetables (green and red pepper, tomato, and lettuce) has reported LODs of 0.01 to 15 mg/kg (ave. 1.5) for tartaric acid, 0.3 to 12 mg/kg (ave. 3.5) for malic acid, 0.13 to 7.8 mg/kg (ave. 2.5) for citric acid, and 0.01 to 0.22 mg/kg (ave. 0.07) for

succinic acid as all fruits and vegetables (Flores et al. 2012), which are comparable with the LODs we obtained using our LC-MS/MS method. Thus, we conclude that our LC-MS/MS method is sensitive enough for the analysis of our targeted organic acids in honey.

Repeatability

The intra-day RSD and inter-day RSD values for the five organic acids ranged from 2.6 to 9.7% and from 2.2 to 14.5%, respectively (Table 4). In the LC-MS/MS-based analysis of fruits and vegetables mentioned in the previous section (Flores et al. 2012), the intra-day RSD and inter-day RSD values of peak area ranged from 0.5 to 1.8% and from 2.9 to 6.0%, respectively, which are lower than the values we obtained in the present study. Wang et al. (2015) have proposed that intra-day RSD and inter-day RSD values within 15% are maximum accepted value, and the values from the present study are within that limit. Thus, we conclude that our LC-MS/MS method has satisfactory reliability and reproducibility for the quantification of our targeted organic acids in honey.

Honey Sample Analysis

Table 5 shows the results for the quantification of gluconic acid, tartaric acid, malic acid, citric acid, and succinic acid in

Table 2 Linear ranges (mg/kg, mg organic acid/kg honey sample), linear equations, and the coefficient of determination (R^2) for the target organic acids

	Linear range (mg/kg)	Linear equation	R^2
Gluconic acid	0.5–20	$f(x) = 67,574x + 18,657$	0.999
Tartaric acid	0.5–10	$f(x) = 14,087x + 1509$	0.998
Malic acid	0.5–10	$f(x) = 63,116x + 37,863$	0.985
Citric acid	0.5–10	$f(x) = 94,360x - 957$	0.998
Succinic acid	0.5–10	$f(x) = 11,458x + 2160$	0.998

Table 3 Limits of detection (LOD) and quantification (LOQ) for the target organic acids in honeys (mg/kg)

No.	Gluconic acid		Tartaric acid		Malic acid		Citric acid		Succinic acid	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
1	0.09	0.31	0.04	0.14	0.11	0.37	0.04	0.14	0.15	0.48
2	0.11	0.37	0.01	0.04	0.02	0.07	0.02	0.08	0.04	0.13
3	0.09	0.29	0.04	0.13	0.25	0.82	0.06	0.21	0.03	0.09
4	0.06	0.20	0.03	0.09	0.08	0.27	0.05	0.16	0.10	0.34
5	0.10	0.33	0.02	0.08	0.01	0.05	0.04	0.13	0.02	0.08
6	0.03	0.11	0.005	0.02	0.04	0.14	0.04	0.13	0.02	0.08
7	0.03	0.11	0.01	0.03	0.03	0.09	0.03	0.09	0.09	0.29
8	0.17	0.56	0.01	0.04	0.05	0.17	0.05	0.16	0.06	0.20
9	0.18	0.60	0.04	0.12	0.70	2.32	0.03	0.09	0.06	0.21
10	0.11	0.38	0.03	0.11	0.07	0.22	0.05	0.18	0.05	0.15
11	0.15	0.50	0.07	0.22	0.05	0.17	0.03	0.10	0.15	0.49
12	0.43	1.44	0.03	0.11	0.06	0.22	0.09	0.30	0.08	0.26
13	0.05	0.15	0.05	0.16	0.05	0.18	0.03	0.11	0.05	0.18
14	0.12	0.42	0.09	0.29	0.02	0.06	0.05	0.17	0.24	0.79
15	0.15	0.50	0.07	0.23	0.05	0.18	0.04	0.14	0.06	0.21
16	0.06	0.19	0.06	0.21	0.08	0.27	0.06	0.21	0.08	0.26
17	0.09	0.29	0.01	0.05	0.02	0.05	0.02	0.07	0.10	0.32
18	0.06	0.20	0.01	0.02	0.04	0.13	0.08	0.27	0.02	0.08
19	0.17	0.57	0.03	0.09	0.06	0.21	0.11	0.35	0.12	0.40
20	0.17	0.56	0.03	0.08	0.03	0.11	0.06	0.19	0.11	0.38
21	0.25	0.84	0.02	0.06	0.05	0.15	0.02	0.08	0.05	0.18
22	0.10	0.34	0.04	0.12	0.10	0.32	0.13	0.44	0.22	0.72
23	0.08	0.26	0.03	0.10	0.08	0.26	0.16	0.52	0.13	0.43
24	0.09	0.31	0.02	0.06	0.14	0.47	0.03	0.10	0.14	0.46
25	0.21	0.71	0.03	0.10	0.24	0.79	0.24	0.81	0.02	0.07
Ave	0.13	0.42	0.03	0.11	0.10	0.32	0.06	0.21	0.09	0.29
Min	0.03	0.11	0.005	0.02	0.01	0.05	0.02	0.07	0.02	0.07
Max	0.43	1.44	0.09	0.29	0.70	2.32	0.24	0.81	0.24	0.79

Abbreviations: *Ave* average, *Min* minimum, *Max* maximum

Table 4 Intra-day relative standard deviation (RSD) (%) and inter-day RSD (%) for the target organic acids at concentrations of 1 and 5 mg/kg

Organic acid	Concentration	Intra-day RSD (%)[a]	Inter-day RSD (%)[b]
Gluconic acid	1	2.6	9.8
	5	2.9	4.0
Tartaric acid	1	4.1	3.3
	5	3.7	3.7
Malic acid	1	7.3	4.0
	5	2.7	2.2
Citric acid	1	5.5	8.6
	5	3.8	4.2
Succinic acid	1	9.7	14.5
	5	4.1	5.4

[a] Intra-day, $n = 10$; [b] Inter-day, $n = 3$

Table 5 Geographical origins of the honey samples and their organic acid concentrations (mg/kg) as determined by LC-MS/MS ($n = 3$)

Sample	Country	Gluconic acid	Tartaric acid	Malic acid	Citric acid	Succinic acid
1	China	1682.9 ± 66.6	n.d.	23.4 ± 0.6	31.9 ± 3.6	11.7 ± 3.9
2	China	1629.6 ± 68.5	n.d.	15.6 ± 0.6	n.d.	23.1 ± 0.5
3	China	649.0 ± 34.1	n.d.	262.6 ± 41.2	58.3 ± 6.3	34.8 ± 15.3
4	Japan	1616.7 ± 29.3	n.d.	28.8 ± 2.6	10.6 ± 1.8	n.d.
5	Japan	1337.7 ± 37.4	n.d.	10.3 ± 0.6	11.8 ± 2.3	n.d.
6	Japan	1549.8 ± 54.7	n.d.	33.3 ± 1.2	27.1 ± 0.7	n.d.
7	Japan	1239.1 ± 36.5	n.d.	34.4 ± 1.3	29.3 ± 3.3	n.d.
8	Japan	2748.4 ± 69.1	n.d.	22.7 ± 1.8	6.7 ± 0.8	n.d.
9	Japan	6475.1 ± 315.2	15.0 ± 1.3	1724.4 ± 280.8	307.4 ± 15.2	91.9 ± 9.7
10	Japan	3781.0 ± 110.1	n.d.	36.3 ± 1.8	22.7 ± 2.1	21.2 ± 3.1
11	Japan	2703.5 ± 143.1	n.d.	n.d.	5.7 ± 0.7	n.d.
12	Japan	2364.0 ± 79.4	n.d.	45.1 ± 6.8	27.1 ± 3.1	n.d.
13	Japan	4280.0 ± 95.4	n.d.	80.9 ± 1.2	98.4 ± 1.1	7.9 ± 1.6
14	Spain	3250.3 ± 82.7	n.d.	44.3 ± 4.2	78.9 ± 8.2	n.d.
15	Spain	2877.7 ± 41.1	n.d.	52.2 ± 1.7	89.1 ± 6.9	14.4 ± 7.5
16	Romania	1938.4 ± 115.8	n.d.	25.9 ± 3.4	24.7 ± 4.4	6.8 ± 1.8
17	Canada	1853.2 ± 32.6	n.d.	14.8 ± 1.0	27.7 ± 0.5	8.9 ± 3.9
18	Canada	2165.6 ± 82.9	n.d.	20.6 ± 0.3	31.0 ± 1.1	4.9 ± 0.4
19	USA	5459.5 ± 22.4	9.3 ± 2.9	188.0 ± 10.9	191.5 ± 15.0	64.9 ± 6.9
20	Argentina	3247.9 ± 216.4	n.d.	29.2 ± 2.2	30.5 ± 1.6	n.d.
21	NZ	1842.1 ± 52.3	n.d.	40.0 ± 0.3	6.2 ± 0.6	5.1 ± 4.4
22	NZ	4769.5 ± 216.9	n.d.	110.1 ± 6.1	98.1 ± 2.7	23.9 ± 5.7
23	NZ	4930.7 ± 185.2	2.8 ± 1.5	257.5 ± 8.6	130.0 ± 5.7	39.7 ± 9.1
24	NZ	5048.6 ± 286.1	5.7 ± 1.2	198.3 ± 8.0	129.1 ± 3.0	39.5 ± 5.1
25	NZ	5448.9 ± 264.9	7.2 ± 1.1	267.6 ± 28.3	288.6 ± 42.4	58.6 ± 16.5
Average		2995.6 ± 1602.3	8.0 ± 4.6	148.6 ± 346.4	73.4 ± 84.2	28.6 ± 25.2
Min		649.0	2.8	10.3	5.7	4.9
Max		6475.1	15.0	1724.4	307.4	91.9

Abbreviations: *NZ* New Zealand, *n.d.* not detected, *Min* minimum, *Max* maximum, *error*, ± 1 S.D.

25 honey samples. Gluconic acid was the most abundant organic acid, followed by malic acid and citric acid, and these organic acids were detected in almost all samples. Some samples for succinic acid were detected, and few samples for tartaric acid were detected. The proportion of gluconic acid in the total amount of organic acid ranged from 64.6 to 99.8% (ave. 94.1%), which is comparable with the range (approx. 79% to 98%) reported by Mato et al. (2006b). As statistical analysis, the one-way analysis of variance (ANOVA) was performed for each component for investigating botanical origin and geographic origin. As a result, there was no significant difference in each component between botanical origin and geographic origin.

Gluconic Acid

Gluconic acid was detected in all 25 samples, and the average gluconic acid concentration was 2995.6 ± 1602.3 mg/kg (Table 5). Gluconic acid in honey derives from two main sources: production by the action of bee glucose oxidase on nectar glucose and production by the metabolic activity of certain *Gluconobacter* spp. (Mato et al. 2003). Using capillary zone electrophoresis, ion chromatography, and high-performance liquid chromatography methods, it has been reported that honey contains 1600 to 14,400 mg/kg of gluconic acid (Cherchi et al. 1994; Daniele et al. 2012; Del Nozal et al. 1998; Mato et al. 2006b). The concentrations of gluconic acid in this study were comparable with this previously reported range, except that in sample no. 3. The sample no.3 was the lowest concentration, a significant difference ($p < 0.001$) to other honey samples in this research. It has been reported that heating reduces the amount of gluconic acid in honey (Wang et al. 2004). Thus, the sample no. 3 might be heated or artificially diluted with water. Indeed, this sample was identified in a study using carbon stable isotope ratios as having been adulterated (Kawashima et al. 2019).

Table 6 Coefficients of determination for the correlations between concentrations of the target organic acids in honey

	Gluconic acid	Tartaric acid	Malic acid	Citric acid	Succinic acid
Gluconic acid	—	0.97**	0.56*	0.83**	0.73**
Tartaric acid		—	0.84**	0.77**	0.97**
Malic acid			—	0.73**	0.79**
Citric acid				—	0.91**
Succinic acid					—

$*p < 0.01$, $**p < 0.001$

Acacia honey is reported to contain 1857 ± 458 mg/kg of gluconic acid (Daniele et al. 2012). The present study included three Acacia honeys (sample nos. 6, 7, and 16). The average concentration for gluconic acid in three samples was 1575.8 ± 350.4 mg/kg, which is comparable with the range reported by Daniele et al. (2012). Although the gluconic acids in acacia honey were relatively low to other honey samples, it was shown no statistically significant by the one-way ANOVA.

Malic Acid, Citric Acid, Succinic Acid, and Tartaric Acid

Malic acid was detected in 24 of 25 samples, and the average malic acid concentration was 148.6 ± 346.4 mg/kg (Table 5). It has been reported that the malic acid concentration in honey ranges from 0.0 to 1806 mg/kg (Cherchi et al. 1994; Del Nozal et al. 1998; Haroun et al. 2012; Mato et al. 1998b, 2006b). The malic acid concentrations obtained in the present study were within the previously reported range. However, sample no. 9 showed a significant difference compared with other honeys ($p < 0.001$). In the present study, sample no. 9 was an orange honey. Orange fruit contains malic acid (Fernández-Fernández et al. 2010); therefore, the malic acid detected may have been added in the form of orange extract.

Citric acid was detected in 24 of 25 samples, and the average citric acid concentration was 73.4 ± 84.2 mg/kg (Table 5). It has been reported that the citric acid concentration in honey ranges from 44 to 1184 mg/kg (Cherchi et al. 1994; Daniele et al. 2012; Del Nozal et al. 1998; Haroun et al. 2012; Mato et al. 2006b), and the citric acid concentrations obtained in the present study were within this range. The previous study reported that citric acid concentration can be used as a parameter to differentiate between floral and honeydew honeys (Haroun et al. 2012; Talpay 1988). All of the honey samples in this study were flower honeys, and these values were considered to be reasonable.

Succinic acid was detected in 16 of 25 samples, and the average succinic acid concentration was 28.6 ± 25.2 mg/kg (Table 5). It has been reported that the succinic acid concentration in honey ranges from 12 to 1785 mg/kg (Cherchi et al. 1994; Del Nozal et al. 1998; Haroun et al. 2012; Mato et al. 2006b), and the succinic acid concentrations obtained in the present study were within this range.

Tartaric acid was detected in only 5 samples, and the average tartaric acid concentration was 8.0 ± 4.6 mg/kg. Haroun et al. (2012) reported that tartaric acid concentration ranges from 21.4 to 44.2 mg/kg. In this study, the concentration was somewhat lower than that in the previous study.

Correlations Between Organic Acid Concentrations in Honey and Statistical Analysis

Table 6 shows the correlation matrix for the concentrations of the target organic acids in honey. For gluconic acid, the strongest correlation was found between the amount of gluconic acid and that of citric acid ($R = 0.83$). The correlation was slightly stronger between gluconic acid and succinic acid ($R = 0.73$), followed by that between gluconic acid and malic acid ($R = 0.56$). As described above, gluconic acid is produced from glucose by glucose oxidase, and the amount present depends on the time taken to convert nectar to honey (Mato et al. 2003). In contrast, other organic acids are synthesized from nectar glucose, fructose, and sucrose by the action of worker bee enzymes (Echigo and Takenaka 1974).

In addition, principal component analysis (PCA) of gluconic acid, tartaric acid, malic acid, citric acid, and succinic acid were performed (Supplement Fig. 2). For samples below the lower limit of detection, half the lower limit of quantitation was used. The total information from this PCA is 93.0%, represented by 83.8% for component 1 and 9.2% for component 2. It was found that all compounds can be explained for component 1, since the factor loadings in component 1 for all components were > 0.85. Thus, it has been shown that when the gluconic acid concentration is high, the malic acid, citric acid, and succinic acid concentrations are also usually high, suggesting that these organic acids might also be produced by worker bee enzymes such as glucose oxidase.

We found a very strong correlation between citric acid and succinic acid ($R = 0.91$) and strong correlations between malic acid and succinic acid ($R = 0.79$) and malic acid and citric acid ($R = 0.73$). Because these three organic acids are involved in the tricarboxylic acid (TCA) cycle, these correlations strongly suggest that the activities

of these enzymes in the TCA cycle are important for the presence of these organic acids in honey. For scatterplot matrix of factor scores, manuka honey may be distinguishable from other honey due to high citric acid content (Supplement Fig. 2).

Conclusions

This is the first report of the use of LC-MS/MS to determine the concentrations of organic acids in honey samples. The matrix effect was determined for all of the organic acids and led us to use the standard addition protocol to determine the concentrations of the target organic acids in honey samples. The limit of detection for organic acids was ranged from 0.005 to 0.70 mg/kg. In this study, detection limits were 8 to 31 times higher sensitive than high-performance liquid chromatography method. We then used our LC-MS/MS method to determine the concentrations of the five target organic acids in 25 honey samples. Gluconic acid was found to be the most abundant, followed by malic acid and citric acid. The average gluconic acid concentration was 2995.6 ± 1602.3 mg/kg, and the proportion of gluconic acid to total organic acids ranged from 64.6 to 99.8% (ave. 94.1%). From the correlation and statistical analysis of each component, it is considered that the organic acid is produced by worker bee enzymes such as glucose oxidase and the TCA cycle. The developed method, which has high sensitivity and selectivity, will enable the analysis of more complex and low-concentration components. Therefore, it is considered to be applicable to such as food authenticity/origin, metabolism research, and evaluation of foreign substances in the future.

Compliance with Ethical Standards

Conflict of Interest Momoka Suto declares that she has no conflict of interest. Hiroto Kawashima declares that he has no conflict of interest. Yasunori Nakamura declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

References

Castiñeira A, Peña RM, Herrero C, García-Martín S (2002) Analysis of organic acids in wine by capillary electrophoresis with direct UV detection. J Food Compos Anal 15:319–331. https://doi.org/10.1006/jfca.2002.1056

Cherchi A, Spanedda L, Tuberoso C, Cabras P (1994) Solid-phase extraction and high-performance liquid chromatographic determination of organic acids in honey. J Chromatogr A 669:59–64. https://doi.org/10.1016/0021-9673(94)80336-6

Chinnici F, Spinabelli U, Riponi C, Amati A (2005) Optimization of the determination of organic acids and sugars in fruit juices by ion-exclusion liquid chromatography. J Food Compos Anal 18:121–130. https://doi.org/10.1016/j.jfca.2004.01.005

Commission Decision 2002/657/EC (2002) Official Journal of the European Communities L221:8–36

da Silva PM, Gauche C, Gonzaga LV, Costa AC, Fett R (2016) Honey: chemical composition, stability and authenticity. Food Chem 196:309–323. https://doi.org/10.1016/j.foodchem.2015.09.051

Daniele G, Maitre D, Casabianca H (2012) Identification, quantification and carbon stable isotopes determinations of organic acids in monofloral honeys. A powerful tool for botanical and authenticity control. Rapid Commun Mass Spectrom 26:1993–1998. https://doi.org/10.1002/rcm.6310

Del Nozal MJ, Bernal JL, Marinero P, Diego JC, Frechilla JI, Higes M, Llorente J (1998) High performance liquid chromatographic determination of organic acids in honeys from different botanical origin. J Liq Chromatogr Relat Technol 21:3197–3214

Echigo T, Takenaka T (1974) Production of organic acids in honey by honeybees

Fernández-Fernández R, López-Martínez JC, Romero-González R, Martínez-Vidal JL, Alarcón Flores MI, Garrido Frenich A (2010) Simple LC–MS determination of citric and malic acids in fruits and vegetables. Chromatographia 72:55–62. https://doi.org/10.1365/s10337-010-1611-0

Flores P, Hellín P, Fenoll J (2012) Determination of organic acids in fruits and vegetables by liquid chromatography with tandem-mass spectrometry. Food Chem 132:1049–1054. https://doi.org/10.1016/j.foodchem.2011.10.064

Haroun M, Konar N, Poyrazoglu E, Artik N (2012) Organic acid profiles and contents of Turkish honeydew and floral honeys

Hernandez Y, Lobo M, Gonzalez M (2009) Factors affecting sample extraction in the liquid chromatographic determination of organic acids in papaya and pineapple. Food Chem 114:734–741. https://doi.org/10.1016/j.foodchem.2008.10.021

Horváth K, Molnár-Perl I (1998) Simultaneous GC-MS quantitation of o-phosphoric, aliphatic and aromatic carboxylic acids, proline, hydroxymethylfurfurol and sugars as their TMS derivatives: in honeys. Chromatographia 48:120–126. https://doi.org/10.1007/BF02467527

Kawashima H, Suto M, Suto N (2019) Stable carbon isotope ratios for organic acids in commercial honey samples. Food Chem 289:49–55. https://doi.org/10.1016/j.foodchem.2019.03.053

Lin J-T, Liu S-C, Shen Y-C, Yang D-J (2011) Comparison of various preparation methods for determination of organic acids in fruit vinegars with a simple ion-exclusion liquid chromatography. Food Anal Methods 4:531–539. https://doi.org/10.1007/s12161-011-9204-6

Mato I, Huidobro JF, Cendón V, Muniategui S, Fernández-Muiño MA, Sancho MT (1998a) Enzymatic determination of citric acid in honey by using polyvinylpolypyrrolidone clarification. J Agric Food Chem 46:141–144. https://doi.org/10.1021/jf970418p

Mato I, Huidobro JF, Sánchez MP, Muniategui S, Fernández-Muiño MA, Sancho MT (1998b) Enzymatic determination of L-malic acid in

honey. Food Chem 62:503–508. https://doi.org/10.1016/S0308-8146(97)00166-0

Mato I, Huidobro JF, Simal-Lozano J, Sancho MT (2003) Significance of nonaromatic organic acids in honey. J Food Prot 66:2371–2376. https://doi.org/10.4315/0362-028X-66.12.2371

Mato I, Suárez-Luque S, Huidobro JF (2005) A review of the analytical methods to determine organic acids in grape juices and wines. Food Res Int 38:1175–1188. https://doi.org/10.1016/j.foodres.2005.04.007

Mato I, Huidobro JF, Simal-Lozano J, Sancho MT (2006a) Analytical methods for the determination of organic acids in honey. Crit Rev Anal Chem 36:3–11. https://doi.org/10.1080/10408340500451957

Mato I, Huidobro JF, Simal-Lozano J, Sancho MT (2006b) Rapid determination of nonaromatic organic acids in honey by capillary zone electrophoresis with direct ultraviolet detection. J Agric Food Chem 54:1541–1550. https://doi.org/10.1021/jf051757i

Sandin-Espana P, Mateo-Miranda M, Lopez-Goti C, De Cal A, Alonso-Prados JL (2016) Development of a rapid and direct method for the determination of organic acids in peach fruit using LC-ESI-MS. Food Chem 192:268–273. https://doi.org/10.1016/j.foodchem.2015.07.012

Stinson EE, Subers MH, Petty J, White JW Jr (1960) The composition of honey. V Separation and identification of the organic acids. Arch Biochem Biophys 89:6–12. https://doi.org/10.1016/0003-9861(60)90003-5

Talpay B (1988) Inhaltsstoffe des Honigs-Citronensäure (Citrat) Dtsch Lebensm-Rundsch 84:41–44

Van Eeckhaut A, Lanckmans K, Sarre S, Smolders I, Michotte Y (2009) Validation of bioanalytical LC-MS/MS assays: evaluation of matrix effects. J Chromatogr B 877:2198–2207. https://doi.org/10.1016/j.jchromb.2009.01.003

Verzera A, Campisi S, Zappalà M, Bonaccorsi I (2001) SPME-GC-MS analysis of honey volatile components for the characterization of different floral origin. Am Lab 33:18–21

Wang XH, Gheldof N, Engeseth NJ (2004) Effect of processing and storage on antioxidant capacity of honey. J Food Sci 69:FCT96–FC101

Wang S, Fan CQ, Wang P (2015) Determination of ultra-trace organic acids in Masson pine (Pinus massoniana L.) by accelerated solvent extraction and liquid chromatography-tandem mass spectrometry. J Chromatogr B 981-982:1–8. https://doi.org/10.1016/j.jchromb.2014.12.011

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