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ARTICLE

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Analysis of Organic Acids in Fruit Juices by Liquid Chromatography−Mass Spectrometry: An Enhanced Tool for Authenticity Testing

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ABSTRACT: Organic acid analysis plays a fundamental role in the testing of authenticity of fruit juices. Analytical methods used routinely for organic acids suffer from poor reproducibility, often give false positives/negatives for tartaric acid, and do not offer the possibility of analyte confirmation. There are conflicting reports in the literature on the presence/absence of tartaric acid in pomegranate juice, a potential indicator of adulteration with grape juice. In this work, a method based on stable isotope dilution liquid chromatography−tandem mass spectrometry is described for citric, malic, quinic, and tartaric acid in fruit juices. Validation data including precision and recovery in six types of juice are presented. Tartaric and quinic acids were confirmed in pomegranate juice at concentrations of 1−5 and ∼1 mg/L, respectively. These concentrations are much lower than those resulting from adulteration with grape juice and apple juice, respectively, at the 5% level. A separate method for isocitric acid in orange juice based on the single standard addition method is also described.

KEYWORDS: authenticity, adulteration, liquid chromatography−mass spectrometry, organic acids, pomegranate juice

INTRODUCTION

Adulteration of fruit juices is a common occurrence in the marketplace.[1] Organic acid analysis plays a fundamental role in the testing of authenticity of fruit juices. Tartaric acid is usually considered an indicator of grape juice addition to a more expensive juice. Similarly, excess malic and/or quinic acid can be used as an indicator of apple juice addition to a more expensive juice. Quinic/citric, quinic/malic, and citric/malic ratios are important in determining the authenticity of cranberry juice.[2] The citric/isocitric ratio is especially important in orange juice, where a ratio >130 suggests dilution corrected by the addition of citric acid.[3]

Analytical methods used routinely for organic acids, such as AOAC official method 986.13,[4] are based on liquid chromatography (reverse phase or ion exchange) coupled to UV detection. Numerous collaborative studies conducted by the Food Industry Analytical Chemists Committee of the Grocery Manufacturers Association have shown that the interlaboratory variability of results generated by these methods is often extremely high. The accurate determination of especially the minor organic acids (e.g., citric in apple, quinic in orange) is often challenging. The quantitation of quinic acid in general suffers from poor reproducibility except for cranberry juice, in which it is a major component. False positives and false negatives are quite common for tartaric acid, which is also outside the scope of AOAC official method 986.13 (unpublished data). Obviously these methods do not offer the possibility of analyte confirmation other than by retention time.

An emerging issue is related to the conflicting reports that have appeared in the literature on the presence of tartaric acid in pomegranate juice. Initial reports of high concentrations of tartaric acid in pomegranates and pomegranate juice[5,6] could not be confirmed by others.[7,8]

To date, extremely limited work has been done on organic acid analysis by liquid chromatography−mass spectrometry (LC-MS) in general,[9−12] and applied to fruit juices in particular.[13,14] Recently, single-stage LC-MS with stable isotope dilution was applied to the analysis of citric, malic, and quinic acids in *Vaccinium* berry standard reference materials.[15]

In this work, a comprehensive evaluation of a stable isotope dilution liquid chromatography−tandem mass spectrometry (LC-MS/MS) approach to organic acid analysis in fruit juices is presented. Additional work is presented related to the confirmation of tartaric acid and quinic acid in pomegranate juice and implications for authenticity testing. Finally, a separate method for isocitric acid in orange juice is briefly explored.

MATERIALS AND METHODS

Chemicals and Reagents. Citric acid anhydrous was purchased from ICN Biomedicals Inc. (Aurora, OH). DL-Isocitric acid trisodium salt (14.9% water, 97% enzymatic purity based on D-isocitrate), L-(−)-malic acid, (1*R*,3*R*,4*R*,5*R*)-(−)-quinic acid (98%), L-tartaric acid (99.5%), formic acid, ammonium acetate, methanol (>99.9%, HPLC grade), sodium hydroxide, and hydrochloric acid (37%) were purchased from Sigma-Aldrich (Milwaukee, WI). Acetonitrile (>99.9%, HPLC grade) was from Fisher Scientific (Fair Lawn, NJ). Citric-2,2,4,4-d_4 acid (98.2 atom %D), (*RS*)-malic-2,3,3-d_3 acid (98.6 atom %D), and (±)-tartaric-2,3-d_2 acid (98 atom %D) were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). (±)-Quinic acid-[$^{13}C_3$]-$^1/_2CH_3CH_2OH$ was synthesized by Isosciences (King of Prussia, PA) and was provided as a gift by the National Institute of Standards and Technology (Gaithersburg, MD).

Fruit Juices. Authentic samples of apple, orange, cranberry, (white and red) grape, and pomegranate juice concentrates were provided by Tree Top Inc. (Selah, WA), The Coca-Cola Co. (Apopka, FL), Oceanspray Cranberries, Inc. (Lakeville/Middleboro, MA), Welch Foods, Inc. (Billerica, MA), and POM Wonderful, LLC (Los Angeles, CA), respectively. Authentic samples of single-strength pomegranate

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Table 1. Multiple Reaction Monitoring (MRM) Transitions Used for Organic Acids (Tartaric, Quinic, Malic, and Citric) and Their Isotopically Labeled Internal Standard Analogues

RT[a] (min)	organic acid	MRM transitions	cone voltage (V)	collision energy (eV)
5.2	tartaric	149.0 > 86.9	30	15
		149.0 > 72.8	30	15
5.2	d_2-tartaric	151.0 > 87.9	30	15
5.6	quinic	191.1 > 84.9	30	20
		191.1 > 92.9	30	20
5.6	3^{13}C-quinic	194.1 > 86.9	30	20
7.1	malic	133.0 > 115.0	20	10
		133.0 > 70.9	20	15
7.1	d_3-malic	136.0 > 117.0	20	10
12.7	citric	191.1 > 86.9	20	20
		191.1 > 110.9	20	12
12.4	d_4-citric	195.1 > 114.0	20	12

[a] RT, retention time.

juice (squeezed from fruit) and commercial single-strength pomegranate juice were provided by the Oregon State University (Corvallis, OR).

All concentrates were diluted to single-strength before analysis (apple, 11.5 °Brix; orange, 11.8 °Brix; cranberry, 7.5 °Brix; grape, 16 °Brix; pomegranate, 16 °Brix).

Preparation of Standards and Samples. A stock solution containing citric, malic, quinic, and tartaric acid each at 1000 mg/L was prepared in laboratory-deionized water. An internal standard stock solution containing the isotopically labeled analogues citric, malic, quinic, and tartaric acid each at 500 mg/L was also prepared in deionized water. Calibration standards containing 5, 25, 100, and 250 mg/L each of citric, malic, quinic, and tartaric acid were prepared by diluting the stock solution with deionized water.

Single-strength juices were diluted in a ratio of 1:10, 1:20, or 1:100 (in most cases) with deionized water up to a final volume of 1 mL. A 1:5 dilution was performed for the analysis of trace levels of tartaric acid in pomegranate juice and quinic acid in red grape and pomegranate juice. For juices analyzed on the HILIC column, dilution was made with acetonitrile/water (50:50). For spiked samples, an appropriate amount of spiking solution (containing either 1000 mg/L or 10000 mg/L organic acids) was added to the juice, and the volume was made up to 1 mL with deionized water.

To 1 mL of standard solution or diluted juice was added 50 μL of the internal standard stock solution. After mixing, cloudy samples were filtered through a 0.45 μm pore size nylon syringe filter before analysis.

All samples (unspiked and spiked) were prepared and analyzed in triplicate. For samples analyzed on the HILIC column, six replicates were prepared and analyzed.

For the analysis of isocitric acid, 100 μL of single-strength orange juice (unspiked or spiked with an appropriate amount of a 1000 mg/L spiking solution) was incubated with 20 μL of 4 N NaOH for 10 min, followed by the addition of 20 μL of 4 N HCl and 860 μL of deionized water. Samples were filtered before analysis as described above. Six replicates (both unspiked and spiked) were prepared.

Instrumentation. An Agilent Technologies 1100 HPLC system interfaced with a Waters Quattro Micro MS/MS instrument equipped with an electrospray ionization source was used.

Chromatographic separation was carried out according to a protocol similar to that described in ref 15. A 250 × 4.6 mm (5 μm) Allure Organic Acids column (Restek Corp., Bellefonte, PA) fitted with a 10 × 4.6 mm (5 μm) guard column at 30 °C was used. Mobile phase was water containing 0.5% formic acid, delivered at 0.7 mL/min. The column effluent was split in a ratio of ~1:1 before the ionization source. The injection volume was 10 μL.

For additional confirmation of tartaric acid and quinic acid in pomegranate juice, a Sequant 150 × 2.1 mm (5 μm) ZIC-HILIC column (The Nest Group Inc., Southborough, MA) fitted with a 20 × 2.1 mm (5 μm) guard column at 30 °C was used. The following mobile phase gradient was employed (A, acetonitrile/water (90:10) containing 0.1% of ammonium acetate; B, water containing 0.1% of ammonium acetate): 0−20 min, 0−55% B at 0.2 mL/min; 20−25 min, 55% B at 0.2 mL/min; 25−38 min, 0% B at 0.6 mL/min; 38−40 min, 0% B at 0.2 mL/min. The column effluent was introduced in the ionization source without splitting. Injection volumes of 10 μL were used.

For the analysis of isocitric acid, the Allure Organic Acids column was used as described above except for the mobile phase, which was water/methanol (85:15) containing 0.5% of formic acid.

Two multiple reaction monitoring (MRM) transitions in the negative ion mode were used for each organic acid for quantitation and confirmation, respectively (Table 1). The dwell time, interchannel delay, and interscan delay were 0.1, 0.02, and 0.1 s, respectively. Other operating parameters were as follows: capillary voltage, 3 kV; source and desolvation temperature, 120 and 350 °C; desolvation and cone gas flow rates, 900 and 50 L/h, respectively.

Quantitation was performed by the internal standard method for citric, malic, quinic, and tartaric acid and by the single standard addition method for isocitric acid.

RESULTS AND DISCUSSION

The Allure Organic Acids column operated with an all-aqueous mobile phase allows for excellent separation of tartaric, quinic, malic, and citric acid (Figure 1). The use of two MRM transitions for each organic acid (Table 1) allows the confirmation of analyte identity in each case. This is especially important when trace levels of tartaric acid and quinic acids are encountered in a juice sample that does not typically contain these organic acids and could be suspected for adulteration with grape juice and apple juice, respectively. Ratios of MRM transitions determined in all juice samples for all organic acids studied were typically within ±10% of the respective ratios measured in standards and well within acceptable limits.[16] The use of isotopically labeled internal standards allowed for accurate quantitation of organic acids even at concentrations as low as 1−5 mg/L (ppm) without any significant matrix effects.

For calibration curves, a quadratic regression model ($1/x$ weighted, origin excluded) gave the best fit over the concentration range studied (5−250 mg/L). The coefficients of determination (r^2) were >0.99 in all cases, and residuals were typically <5%.

Sample preparation consisted of a simple dilution of the single-strength juice with water in a ratio of 1:10, 1:20, or 1:100. In certain selected cases when an organic acid was present at the low mg/L (ppm) level in a juice (e.g., tartaric in pomegranate), a 1:5 dilution was used. The method described here was validated in six different fruit juice matrices (apple, orange, cranberry, white grape, red grape, and pomegranate) covering most organic acid patterns typically encountered in

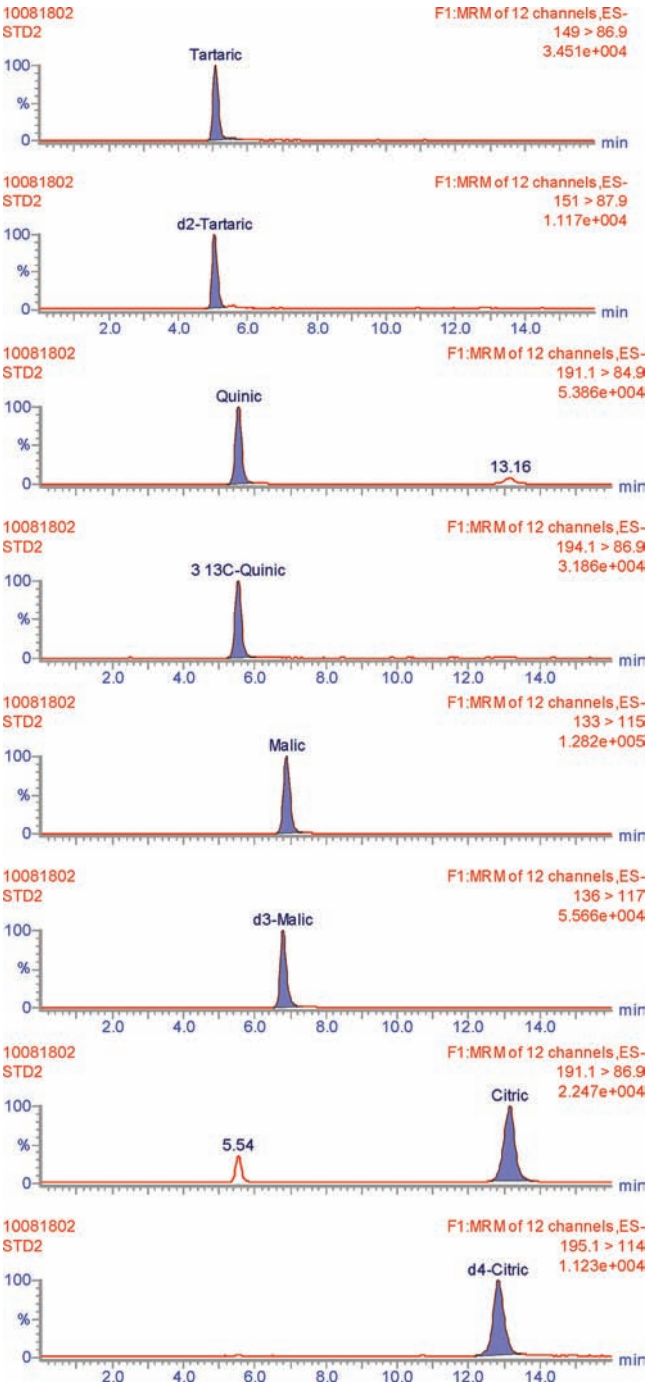

Figure 1. Multiple reaction monitoring (MRM) chromatograms of organic acids (tartaric, quinic, malic, and citric) and their isotopically labeled analogues in a 25 mg/L standard solution. Analysis was performed on the Allure Organic Acids column with water containing 0.5% formic acid as mobile phase.

juice analysis. Precision was determined by carrying out analyses in triplicate for each organic acid in each type of juice. Accuracy was determined by spiking each organic acid into each type of juice matrix at a comparable concentration to that found in the respective juice and calculating recovery (total). In those instances when a certain organic acid was not detected in a given matrix, spiking was carried out at the low end of the calibration curve (50 mg/L in the juice, corresponding to 5 mg/L after 1:10 dilution). Spiking experiments were also carried out in triplicate. The validation data are summarized in Table 2.

Relative standard deviations were <5% in most cases. Slightly worse precision was encountered for citric acid in certain matrices (9.4 and 7.2% in apple and cranberry juices, respectively). This is due to the slight separation (by ca. 0.3 min) of citric acid from its isotopically labeled analogue d_4-citric acid in the chromatogram, resulting in less than perfect compensation of matrix effects. This separation can be overcome by using 10% methanol in the mobile phase, which in turn results in coelution of tartaric and quinic acids. Average recoveries were in the 92−111% range, showing the absence of significant matrix effects. The method allows for accurate quantitation of both major (e.g., citric in orange, malic in apple) and minor organic acids (e.g., citric in apple, quinic in orange) in fruit juices. The presence and confirmation of low levels of tartaric acid (1−5 mg/L) and quinic acid (∼1 mg/L) in pomegranate juice are reported here for the first time.

Limits of detection (LOD) and limits of quantitation (LOQ) were determined for those organic acids for which a blank matrix was available. The LODs and LOQs corresponding to signal-to-noise ratios (root-mean-square) of 3:1 and 10:1, respectively, were estimated after spiking each blank juice with the respective organic acids at 5 mg/L and measuring the resulting signal-to-noise ratios after 1:10 dilution of the juice. The LOD and LOQ for tartaric acid in apple, orange, and cranberry juice were 0.3 and 1 mg/L, respectively. The LOD and LOQ for quinic acid in white grape juice were 0.2 and 0.7 mg/L, respectively. In most other cases and in practical juice analysis, concentrations of organic acids are encountered at levels well above the LOQ and the determination of the LOD and LOQ is of no particular interest.

Due to recent conflicting reports in the literature on the presence of tartaric acid in pomegranate juice and the numerous instances of adulteration reported for this increasingly popular juice,[7] additional work was undertaken to settle the controversy surrounding the presence of tartaric acid in pomegranate juice, which can be used as an indicator of adulteration with grape juice.

Besides the MRM transitions described in Table 1, three additional transitions were selected for confirmation of tartaric acid: 149.0 > 42.9, 149.0 > 74.9, and 149.0 > 102.9. Four different ion ratios were calculated for tartaric acid (present at 3.4 mg/L) in a sample of pomegranate juice (after 1:5 dilution with water) and also for tartaric acid in a 0.5 mg/L standard solution. Six replicates of both the pomegranate juice and the standard solution were analyzed. Average ion ratios calculated in the pomegranate juice were within ±11% of the respective average ion ratios in the standard solution and well within acceptable limits.[16] For additional confirmation, a HILIC column with orthogonal selectivity was used. The retention time of tartaric acid in pomegranate juice on the HILIC column was in close agreement with that in the standard solution. Moreover, spiking tartaric acid in the pomegranate juice resulted in a corresponding increase in peak intensity with no peak separation (Figure 2). Similar confirmation was carried out for quinic acid using two additional MRM transition (191.1 > 87.0 and 191.1 > 127.1) and three different ion ratios on both columns (data not shown).

A mini-survey of tartaric acid in six samples of pomegranate juice (squeezed from fruit) and six commercial juices (deemed authentic) resulted in positive identification in all samples at concentrations in the 1−5 mg/L range. Four additional commercial juices contained tartaric acid at much higher levels ranging from 67 to 380 mg/L and were likely adulterated with grape juice. Figure 3 shows MRM chromatograms of tartaric acid

Table 2. Method Validation Data (Precision and Accuracy) for Organic Acids in Six Types of Juice

			analysis result (n = 3)			recovery (%) (n = 3)	
juice type	organic acid	DF[a]	mean ± SD[b]	RSD[c] (%)	spike	mean ± SD	RSD (%)
apple	citric (mg/L)	100	54.0 ± 5.1	9.4	50	93.8 ± 8.2	8.7
	malic (g/L)	100	3.80 ± 0.05	1.3	5	97.4 ± 6.8	7.0
	quinic (g/L)	10	0.406 ± 0.005	1.1	0.5	102.0 ± 1.9	1.9
	tartaric (mg/L)	10	ND[d]		50	99.3 ± 2.6	2.6
orange	citric (g/L)	100	6.65 ± 0.27	4.1	5	102.1 ± 7.9	7.7
	malic (g/L)	100	1.947 ± 0.006	0.3	5	101.7 ± 3.1	3.0
	quinic (mg/L)	10	74.6 ± 3.2	4.3	50	100.3 ± 2.1	2.1
	tartaric (mg/L)	10	ND		50	95.7 ± 0.9	0.9
cranberry	citric (g/L)	100	12.63 ± 0.91	7.2	10	91.8 ± 3.6	3.9
	malic (g/L)	100	7.51 ± 0.30	4.0	10	108.7 ± 4.1	3.8
	quinic (g/L)	100	10.80 ± 0.30	2.8	10	100.5 ± 3.8	3.8
	tartaric (mg/L)	10	ND		50	103.1 ± 3.2	3.1
white grape	citric (g/L)	10	0.313 ± 0.015	4.7	0.5	110.5 ± 4.6	4.2
	malic (g/L)	10	0.906 ± 0.008	0.8	0.5	97.4 ± 3.6	3.6
	quinic (mg/L)	10	ND		50	102.8 ± 1.4	1.4
	tartaric (g/L)	10	0.902 ± 0.036	4.0	1	96.1 ± 3.7	3.8
red grape	citric (g/L)	20	0.327 ± 0.013	3.9	1	101.1 ± 6.8	6.8
	malic (g/L)	20	2.227 ± 0.031	1.4	1	101.4 ± 2.9	2.8
	quinic (mg/L)	5 (10)	3.63 ± 0.10	2.8	50	100.0 ± 1.6	1.6
	tartaric (g/L)	20	0.795 ± 0.020	2.5	1	101.4 ± 3.4	3.3
pomegranate	citric (g/L)	100	12.33 ± 0.21	1.7	10	101.1 ± 6.8	6.8
	malic (g/L)	10	0.820 ± 0.015	1.8	1	101.4 ± 2.9	2.8
	quinic (mg/L)	5 (10)	1.457 ± 0.015	1.0	50	100.0 ± 1.6	1.6
	tartaric (mg/L)	5 (10)	3.41 ± 0.16	4.7	50	101.4 ± 3.4	3.3

[a] DF, dilution factor used for analysis. [b] SD, standard deviation. [c] RSD, relative standard deviation. [d] ND, not detected.

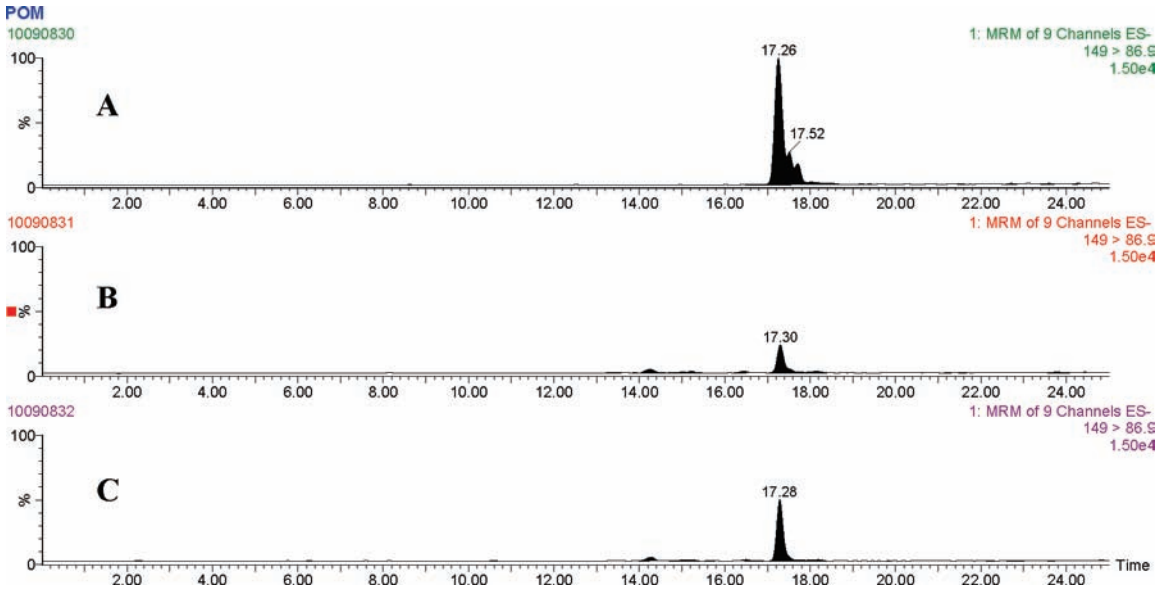

Figure 2. MRM chromatograms of tartaric acid (eluting at 17.3 min) on the Sequant ZIC-HILIC column: A, tartaric acid standard (5 mg/L); B, pomegranate juice; C, pomegranate juice spiked with tartaric acid at 5 mg/L. Juice samples were diluted in a ratio of 1:5 before analysis.

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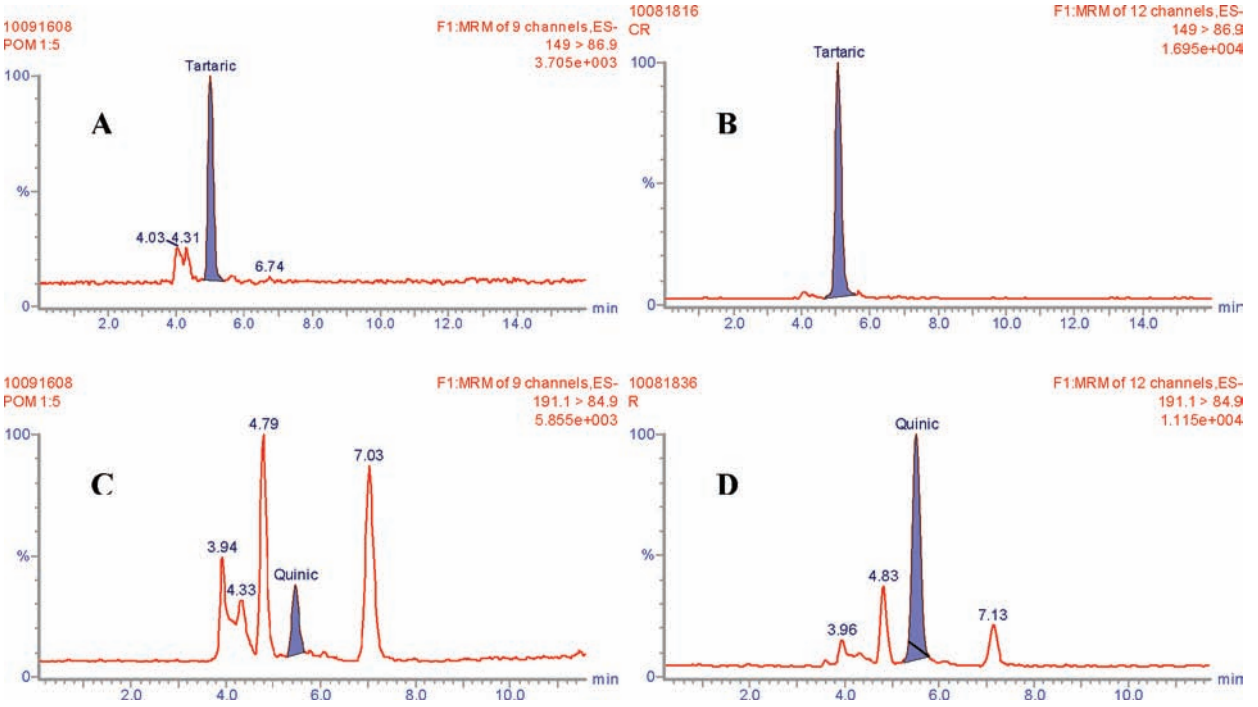

Figure 3. MRM chromatograms of tartaric acid at 3.5 mg/L in an authentic pomegranate juice (A) and in a suspect pomegranate juice at 67 mg/L (B) and of quinic acid at 1.0 mg/L in an authentic pomegranate juice (C) and at 32 mg/L in a suspect pomegranate juice (D). Juices were diluted in a ratio of 1:5 before analysis. Analysis was performed on the Allure Organic Acids column with water containing 0.5% formic acid as mobile phase.

Table 3. Multiple Reaction Monitoring (MRM) Transitions Used for Isocitric Acid

RT[a] (min)	organic acid	MRM transitions	cone voltage (V)	collision energy (eV)
4.7	isocitric	191.1 > 72.9	20	20
		191.1 > 117.0	20	15

[a] RT, retention time.

in both an authentic pomegranate juice and one that is suspected of adulteration with grape juice. In a hypothetical scenario in which authentic pomegranate juice containing 5 mg/L of tartaric acid is adulterated with 5% (vol) of low-tartaric grape juice containing 1000 mg/L of tartaric acid, the resulting juice would contain 55 mg/L of tartaric acid, well above naturally occurring levels and comparable to levels found in the commercial adulterated juices.

A mini-survey of quinic acid in six samples of pomegranate juice (squeezed from fruit) and nine commercial juices (deemed authentic) resulted in positive identification in all samples at ca. 1 mg/L. One additional commercial juice contained quinic acid at a much higher level, 32 mg/L, and was likely adulterated with apple juice. Figure 3 shows MRM chromatograms of quinic acid in both an authentic pomegranate juice and one that is suspected of adulteration with apple juice. In a hypothetical scenario in which authentic pomegranate juice containing 1 mg/L of quinic acid is adulterated with 5% (vol) of apple juice containing 500 mg/L of quinic acid, the resulting juice would contain 26 mg/L of quinic acid, well above naturally occurring levels and comparable to levels found in the commercial adulterated juice. Quinic acid can be especially useful for the detection of adulteration of pomegranate juice with apple juice because other analytes used routinely for the detection of apple juice (malic acid, sorbitol) are also present in pomegranate juice.[7]

For the analysis of isocitric acid (mostly in orange juice), an isotopically labeled internal standard is not currently available. For purity verification, a 100 mg/L solution of isocitrate in water prepared from the commercially available DL-isocitric acid trisodium salt was assayed by using the enzymatic method (Boehringer Mannheim/R-Biopharm test kit for D-isocitric acid, Darmstadt, Germany), and the result (97 mg/L) was in good agreement with the expected value, given the enzymatic purity of the material (97% based on D-isocitrate).

Sample preparation for isocitric analysis requires a hydrolysis step to release isocitric acid from its bound forms (lactone, esters), and a modification of the Wallrauch method[17] was used.

On the Allure Organic Acids column isocitric acid largely coelutes with the much larger malic acid peak. To ensure a more reproducible overlap of the two peaks and to shorten analysis time, 15% methanol was added to the mobile phase. Two MRM transitions were used for the quantitation and confirmation of isocitric acid (Table 3).

For the analysis of isocitric acid in orange juice the single standard addition method was used. First, the juice was analyzed as such, followed by a spiked sample. The spiking level was selected in such a way as to result in a 2−3-fold increase in the peak intensity of isocitric acid (Figure 4). Six replicates of two orange juices were analyzed according to the method described above. Concentrations of isocitric acid were found to be 75.8 ± 8.4 and 223 ± 27 mg/L, with corresponding relative standard deviations of 11 and 12%, respectively. The performance of the method could be enhanced in the future if an isotopically labeled analogue of isocitric acid becomes commercially available.

The work described here is the first comprehensive evaluation of stable isotope dilution LC-MS/MS as applied to the analysis of organic acids in fruit juices for compositional studies and authenticity

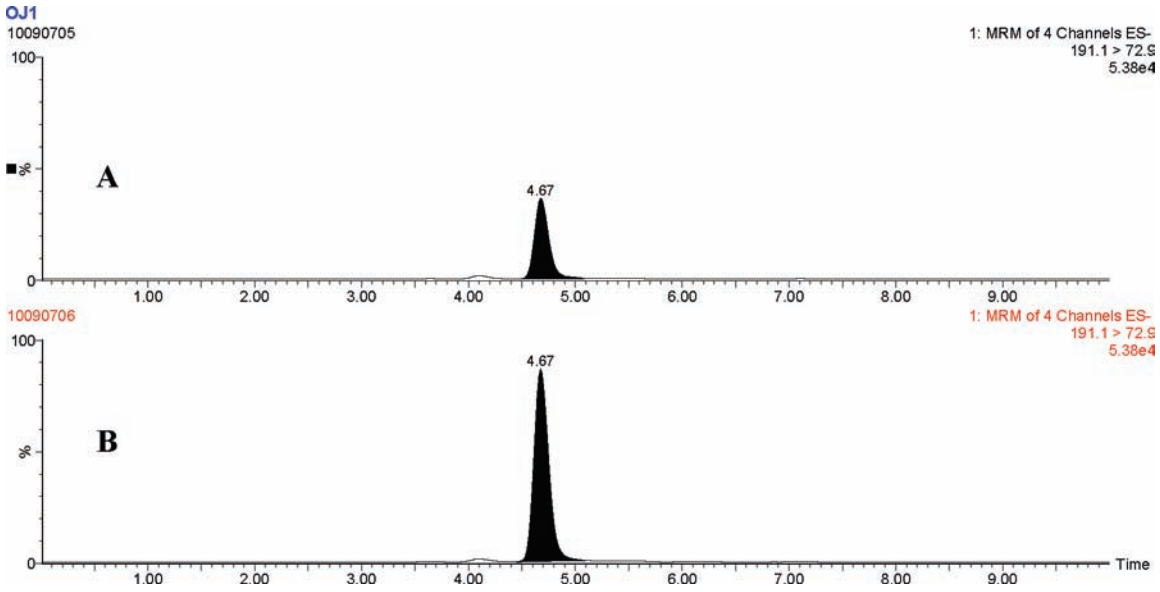

Figure 4. MRM chromatogram of isocitric acid (eluting at 4.7 min) on the Allure Organic Acids column: A, orange juice; B, orange juice spiked with isocitric acid at 100 mg/L. Samples were diluted in a ratio of 1:10 before analysis. Mobile phase is water/methanol (85:15) containing 0.5% formic acid.

determination. It also presents data supporting the unambiguous confirmation of low mg/L (ppm) levels of tartaric acid and quinic acid in pomegranate juice, with important implications for authenticity studies. To our knowledge, this is the first LC-MS-based approach reported in the literature for the analysis of isocitric acid.

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