APPLICATION NOTE

No. 1157

Determination of Organic Acids in Kombucha Using a High-Pressure Ion Chromatography System

Beibei Huang, Jingli Hu, and Jeffrey Rohrer

Keywords
Dionex IonPac AS11-HC-4µm column, Dionex ICS-5000+, HPIC, Suppressed conductivity

Goal

To develop an accurate method for determining organic acids in kombucha using a high-pressure ion chromatography (HPIC) system with suppressed conductivity detection.

Introduction

Kombucha is any of a variety of fermented, lightly effervescent sweetened black or green tea drinks that are commonly intended as functional beverages. It is uniquely fermented simultaneously with yeasts and bacteria in an aerobic environment. In recent years, kombucha has become popular in the U.S. due to its reported health benefits. The reported beneficial effects of kombucha are attributed to the presence of tea polyphenols, gluconic acid, glucuronic acid, lactic acid, vitamins, amino acids, antibiotics, and a variety of other micronutrients produced during fermentation.[1]

Sucrose is the most common carbon source in kombucha fermentation. During the fermentation process, it is generally believed that invertase in yeasts hydrolyzes sucrose into glucose and fructose and produces ethanol via glycolysis, with a preference for fructose as a substrate. Acetic acid bacteria use glucose to produce gluconic acid and ethanol to produce acetic acid.

The determination of organic acids plays an important role in revealing the beneficial effects of kombucha. For example, gluconic acid is associated with detoxification, most notably for heavy metals. Organic acids also affect the flavor and taste of the drink. Therefore, it is necessary to monitor the organic acid profiles of kombucha for product quality purposes.

Several methods have been used to determine organic acids in kombucha, e.g., HPLC with UV detection, etc.[2,3] However, several organic acids have poor UV absorption

and therefore lack sufficient sensitivity for detection. In addition, other components commonly present in kombucha samples, such as sugars and phenolic compounds, are either at much higher concentrations or have a much higher UV absorption, and can, therefore, interfere with the detection of target analytes.

In contrast, virtually all carboxylic acids ionize sufficiently; therefore, ion chromatography (IC) with suppressed conductivity detection is the technique of choice to separate a large variety of organic acids with inorganic anions and detect them with high sensitivity while minimizing the sugar and polyphenol interferences. The Thermo Scientific™ Dionex™ IonPac™ AS11-HC-4µm anion-exchange column is a high resolution, high-capacity column optimized for separating organic acids in complex matrices, and therefore ideal for analysis of kombucha samples.

This work used the properties of the 4-µm-particle-size Dionex IonPac AS11-HC-4µm column, combined with the Thermo Scientific™ Dionex™ ICS-5000⁺ HPIC system, to produce a high-resolution separation of organic acids and inorganic anions in kombucha samples, enabling accurate determinations of those analytes.

Equipment

- Thermo Scientific Dionex ICS-5000⁺ HPIC system, including:
  - Dionex ICS-5000⁺ SP/DP Pump module
  - Dionex ICS-5000⁺ EG Eluent Generator module with high-pressure degasser module
  - Dionex ICS-5000⁺ DC Detector/Chromatography module
- Thermo Scientific™ Dionex™ AS-AP Autosampler with sample syringe, 250 µL (P/N 074306) and buffer line, 1.2 mL (P/N 074989)
- Thermo Scientific™ Dionex™ EGC 500 KOH Eluent Generator Cartridge (P/N 075778)
- Thermo Scientific™ Dionex™ CR-ATC 500 Continuously Regenerated Anion Trap Column (P/N 075550)
- Thermo Scientific™ Dionex™ AERS 500 Suppressor (Dionex AERS 500 (2 mm), P/N 082541)
- 4 L water bottle (P/N 039164)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software version 7.2

Reagents and Standards

Reagents

Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better filtered through a 0.2 µm filter immediately before use.

Standards

- Acetic Acid, Glacial (Certified ACS Plus), Fisher BioReagents (Fisher Scientific P/N BP 2401-500)
- Gluconic acid, sodium salt, 98%, Acros Organics (Fisher Scientific P/N AC 181390010)
- D(+)-Glucuronic acid, sodium salt, monohydrate, 99%, Acros Organics (Fisher Scientific P/N AC 204571000)
- L(-)-Malic Acid, 99% (Fisher Scientific P/N AC15059)
- Pyruvic Acid, Sodium Salt, +99%, Acros Organics (Fisher Scientific P/N AC 132151000)
- Succinic Acid (Crystalline/Certified), Fisher Chemical (Fisher Scientific P/N A294-500)
- Oxalic Acid Dihydrate (Crystalline/Certified ACS), Fisher Chemical (Fisher Scientific, P/N A219-250)
- Citric Acid Anhydrous, Crystalline, USP (Fisher Scientific P/N A95)
- Methanol (Optima™ LC/MS), Fisher Chemical (Fisher Scientific P/N A456-1)

Consumables

- Vial Kit, 10 mL Polystyrene with Caps and Blue Septa (P/N 074228)
- Thermo Scientific™ Nalgene™ Syringe Filters, PES, 0.2 µm (Fisher Scientific P/N 09-740-61A)
- AirTite All-Plastic Norm-Ject™ Syringes, 5 mL, Sterile (Fisher Scientific P/N 14-817-28)
- Thermo Scientific Nalgene 1000 mL, 0.2 µm Nylon Filter Units (P/N 09-740-46)

Samples*

- Kombucha Sample A
- Kombucha Sample B
- Kombucha Sample C

* Samples were purchased from a local store.

Conditions	
Columns:	Dionex IonPac AG11-HC-4µm Guard, 2 × 50 mm (P/N 078036) Dionex IonPac AS11-HC-4µm Analytical, 2 × 250 mm (P/N 078035)
Eluent Source:	Dionex EGC 500 KOH Eluent Generator Cartridge with CR-ATC 500
Eluent A:	DI Water
Eluent B:	Methanol (CH_3OH)

Gradient	
Time (min)	KOH (mM)
-2.000	1
0.000	1
10.070	1
10.071	1
24.000	15
24.010	15
35.000	27
40.000	60
44.000	60
44.010	1
45.000	1

Preparation of Solutions and Reagents

Deionized (DI) water with 18 MΩ-cm resistivity or better was used for eluent and standard preparation and for diluting samples. Individual stock standard solutions of 1000 mg/L were prepared gravimetrically from the reagents and DI water. A mixed standard solution was prepared by diluting the individual stock standard solutions into a 100 mL volumetric flask with DI water. Calibration standards were prepared similarly by diluting the stock standards in DI water. Twenty compounds and their masses, listed in Table 1, were used to prepare 100 mL of 1000 mg/L stock solution.

Methanol was degassed by applied ultrasonic agitation. Degassed methanol was added to a second 1 L eluent bottle on Channel B under inert atmosphere and was introduced at the proportioning valve.

Pump	Multi-Step Gradient	
Time (min)	B (%)	Curve
-2.000	0	5
0.000	0	5
10.700	8	5
20.000	8	5
25.000	15	5
31.000	15	5
33.000	8	5
33.010	0	5
45.000	0	5
Flow Rate:	0.45 mL/min	
Injection Volume:	2.5 µL (full loop)	
Temperature:	45 °C (column compartment), 35 °C (detector compartment)	
System Backpressure:	~4100 psi (1 mM KOH/0% CH_3OH), ~4800 psi (20 mM KOH/15% CH_3OH)	
Detection:	Suppressed Conductivity, Dionex AERS 500 Anion Electrolytically Regenerated Suppressor (2 mm), AutoSuppression, external water mode, external water flow rate (equal to the eluent flow rate)	
Background Conductance:	~ 0.5 µS	
Run Time:	47 min	

Table 1. Amounts of compounds used to prepare 100 mL of 1000 mg/L stock solutions.

Anion	Compound	Mass (mg)
Quinate	Quinic Acid	100.00
Fluoride	Sodium Fluoride	221.01
Lactate	Lactic Acid	100.00
Acetate	Acetic Acid	100.00
Propionate	Sodium Propionate	129.67
Pyruvate	Pyruvic Acid, Sodium Salt	124.96
Gluconate	Gluconic Acid, Sodium Salt	112.36
Glucuronate Sodium Salt Monohydrate	Glucuronic Acid	120.60
Bromide	Sodium Bromide	128.77
Chloride	Sodium Chloride	164.85
Nitrite	Sodium Nitrite	149.96
Sulfate	Sodium Sulfate	147.87
Nitrate	Sodium Nitrate	137.08
Phosphate	Sodium Phosphate, Monobasic	126.33
Succinate	Succinic Acid	100.00
Malate	Malic Acid, Disodium Salt	132.78
Tartrate	Tartaric Acid	100.00
Malonate	Malonic Acid, Disodium Salt	142.23
Oxalate	Oxalic Acid Dihydrate	140.03
Citrate	Citric Acid	100.00

Sample Preparation

Centrifuge kombucha samples at 6500–7500 g for 15 min; pass the supernatant through a Nalgene syringe filter (0.2 µm) and dilute 1:20 with DI water prior to analysis.

Recovery Study

Three organic acids, acetate, succinate, and citrate, were selected for the recovery study. These three are major organic acids found in kombucha, and represent monovalent, divalent, and trivalent organic acids. They are distributed across the early, middle, and late retention time regions of the separation. To be certain that our measurement was accurate, the sample dilutions (1:20) were spiked with known amounts of stock solution.

System Preparation and Configuration

To achieve the best chromatography with HPIC, it is important to use high-pressure connectors and ferrules for all connections prior to the suppressor. The high-pressure Dionex ICS-5000+ HPIC system is designed to operate from 2000 to 5000 psi when using eluent generation. In most cases, the installation instructions are the same when running eluent either with or without methanol. Only the differences are highlighted here for emphasis.

The flow diagram in Figure 1 illustrates the plumbing of the consumables and modules of the Dionex ICS-5000+ HPIC system in recycle mode or external water mode. Install and hydrate the Dionex EGC 500 KOH cartridge, Dionex CR-ATC 500 Continuously Regenerated Anion Trap Column, and Dionex AERS 500 suppressor according to the product manual instructions.[4–6] Note: When methanol is added to the eluent stream, the suppressor must be operated in the External Water mode to prevent contamination from methanol hydrolysis and potential damage to the suppressor.

Install and configure the Dionex AS-AP Autosampler in Push Mode. Follow the instructions in the Dionex AS-AP Autosampler Operator's Manual (Document No. 065361) to calibrate the sample transfer line and ensure accurate and precise sample injections.

Configure the pressurized water reservoirs to supply external water for suppressor regeneration. Use at least two 4 L bottles plumbed in tandem to ensure uninterrupted external water delivery during long periods of uninterrupted operation. Fill the reservoirs with DI water and apply 5–15 psi to the reservoir to deliver DI water through the regenerant channel. Ensure that the cap of the reservoir is sealed tightly.

Install the Dionex IonPac AG11-HC-4µm Guard (2 × 50 mm) and the Dionex IonPac AS11-HC-4µm Analytical (2 × 250 mm) columns in the lower compartment of the DC module. After connecting the inlet of the column, pump 30 mM KOH through the column with the outlet directed to waste for at least 30 min before connecting the column outlet to the suppressor using 0.005 in. i.d. PEEK tubing. Keep the lengths of the connective tubing to a minimum.

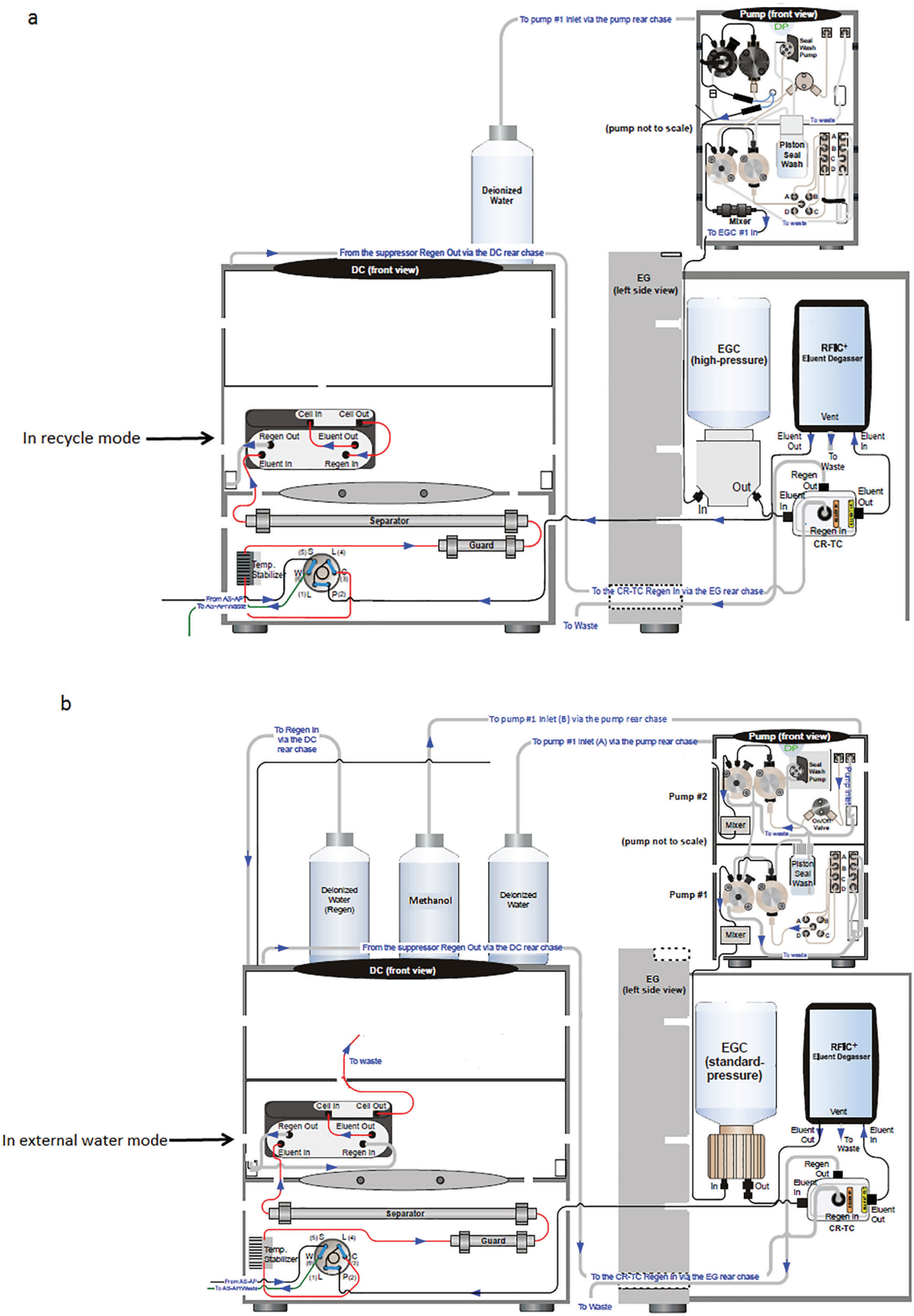


Figure 1. Flow diagram for the Dionex ICS-5000⁺ HPIC system. a) in recycle mode; b) in external water mode.

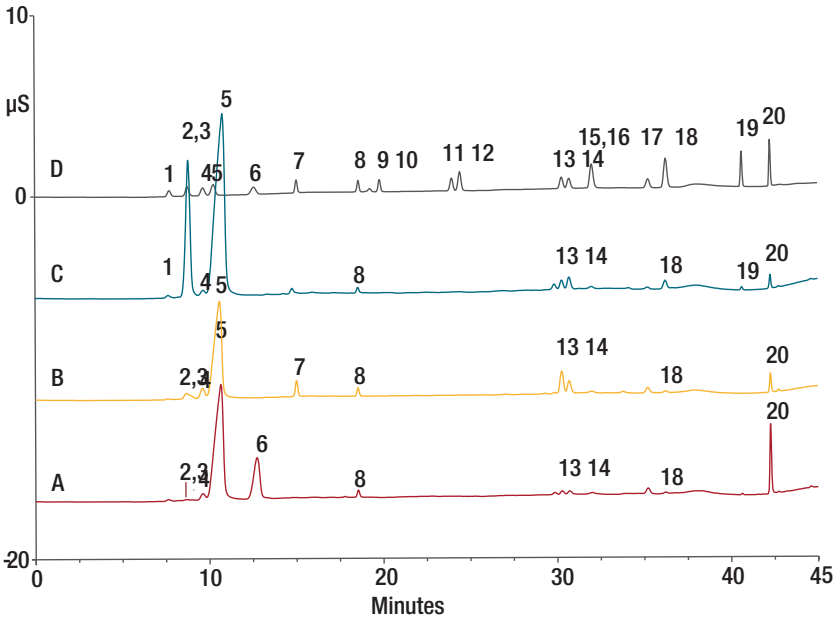
Results and Discussion

Separation

The Dionex IonPac AS11-HC-4µm column is a high resolution, high capacity anion exchange column, providing separations for the best peak identification of a large number of inorganic anions and organic acid anions from a single sample injection. The column is operated in the gradient mode using a hydroxide eluent. Certain organic solvents can be added to the hydroxide eluent to modify the ion exchange process, and thereby column selectivity, or to improve sample solubility. The solvents used must be free of ionic impurities. However, because most solvent manufacturers do not test for ionic impurities, it is important to use the highest grade of solvent available. Currently, several manufacturers are making ultrahigh purity solvents that are compatible with HPLC and spectrophotometric applications. These ultrahigh purity solvents will usually ensure that your chromatography is not affected by ionic impurities in the solvent. In this work, consistent results were achieved using Optima™ LC/MS Grade Solvents.

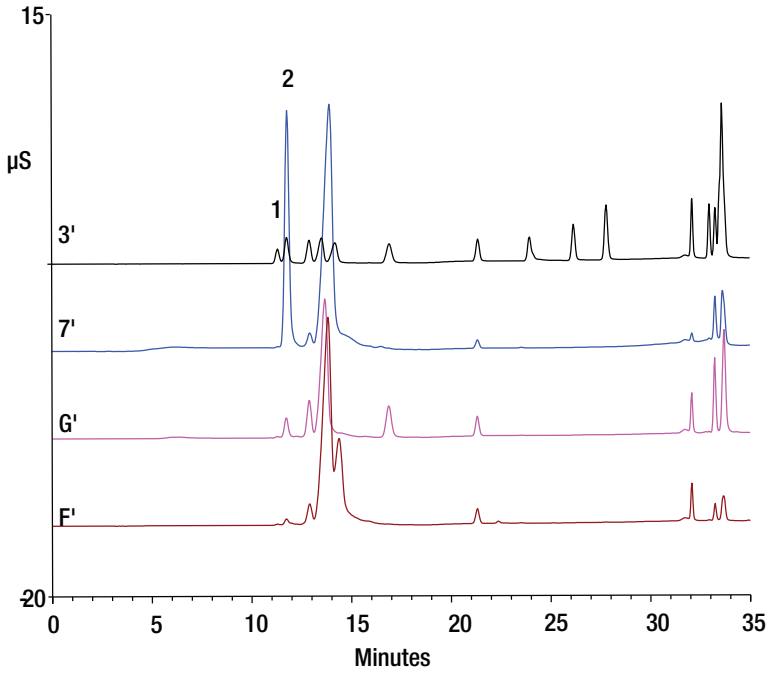
A KOH gradient was used to separate anions of different degrees of affinity for the stationary phase with minimal background shift. Under aqueous eluent conditions, succinate and malate co-elute. In order to improve the separation of malate and succinate, adding methanol to the eluent is recommended. Note that when adding methanol to the eluent stream, the suppressor must be operated in the external water mode. Also note that adding solvent to the aqueous eluent can reduce the peak response with conductivity detection by up to half due to increased eluent viscosity, decreased ionization of organic acids, and lower peak efficiencies. Therefore, only use solvent in the eluent when needed for improved resolution of analytes of interest.

Twenty anions were separated on the Dionex IonPac AS11-HC-4µm column set (Figure 2). The method starts with a low eluent concentration (1 mM KOH) to separate the weakly retained anions, such as quinate, fluoride, gluconate, lactate, and acetate. Using a Dionex IonPac AS11-HC-4µm column set, gluconate and fluoride co-eluted. The addition of methanol did not improve their separation. Gluconate and fluoride were separated on a Dionex IonPac AS19-4µm column set (Figure 3). This analysis revealed that fluoride is either absent or present at very low concentrations in kombucha samples, and therefore gluconate and fluoride separation is not required for this analysis. The Dionex IonPac AS11-HC-4µm column set was used for this analysis because it provided better resolution of more analytes than other anion-exchange columns. After starting at 1 mM KOH, the eluent concentration was gradually increased to elute more strongly retained anions after eluting acetate. Both succinate and malate are present in kombucha samples, so methanol was added to improve resolution for accurate quantification of these compounds. The percentage of methanol was increased to 15% at 25 min and remained at that level for 6 min. To expedite the elution of late-eluting peaks, including phosphate and citrate, no methanol was used from 33–44 min. The eluent condition was restored to the initial condition at 44 min to re-equilibrate the column prior to the next injection. To resolve glucuronate and chloride, the column was operated at 45 °C.



Peaks	Standard	A	B	C
1. Quinate	5 mg/L	2.8 mg/L	0.684 mg/L	1.45 mg/L
2. Fluoride	0.5			
3. Gluconate	5	1.46	9.20	111
4. Lactate	5	2.31	5.58	1.15
5. Acetate	5	1.36	39.0	168
6. Propionate	5	35.4		
7. Pyruvate	5		7.97	
8. Chloride	1.25		0.994	0.505
9. Glucuronate	5			
10. Nitrite	2.5			
11. Bromide	5			
12. Nitrate	5			
13. Succinate	5	1.03	12.8	2.09
14. Malate	5	1.57	7.41	4.27
15. Malonate	5	n.a.	n.a.	n.a.
16. Tartrate	5	n.a.	n.a.	n.a.
17. Sulfate	2.5	1.43	1.08	0.300
18. Oxalate	5	0.319	0.313	1.06
19. Phosphate	7.5	0.298		0.476
20. Citrate	10	16.0	3.85	1.65

Figure 2. Separation of 20 organic and inorganic anions on the Dionex IonPac AS11-HC-4µm column in (A) Kombucha A with 20-fold dilution, (B) Kombucha B with 20-fold dilution, (C) Kombucha C with 20-fold dilution, and (D) a mix of 20 anion standards (complete conditions shown in the Conditions section).



Column:	Dionex AS19-4µm, Analytical, 4 × 250 mm Dionex AG19-4µm, Guard, 4 × 50 mm
Eluent Source:	Dionex EGC 500 KOH cartridge with CR-ATC 500
Gradient:	5 mM KOH (-5-15 min), 5–30 mM (15-25 min), 30–60 mM (25–33 min), 60 mM (33-35 min)
Column Temperature:	50 °C
Flow Rate:	0.8 mL/min
Inj. Volume:	10 µL
Detection:	Suppressed conductivity, Dionex AERS 500 suppressor, 4 mm, AutoSuppression, recycle mode
Samples:	A: Kombucha A with 20-fold dilution; B: Kombucha B with 20-fold dilution; C: Kombucha C with 20-fold dilution; D: a mix of standards, fluoride (0.5 mg/L) and (5 mg/L) gluconate
Peaks:	1. Fluoride 2. Gluconate

Figure 3. Separation of gluconate and fluoride on the Dionex IonPac AS19-4µm column.

Table 2. Method calibration, LODs, and LOQs.

Compound	Range (mg/L)	Coefficient of Determination (r^2)[a]	LOD[b] (mg/L)	LOQ[c] (mg/L)
Gluconate	0.2–150	0.9996	0.042	0.14
Acetate	5–400	0.9997	0.006	0.02
Lactate	1–100	0.9998	0.012	0.04
Pyruvate	1–100	0.9998	0.021	0.07
Succinate	1–100	0.9999	0.057	0.19
Malate	1–100	0.9996	0.020	0.07
Oxalate	0.2–20	1.000	0.027	0.09
Citrate	1–100	0.9996	0.013	0.04

[a]Quadratic fit
[b]LOD=3×S/N
[c]LOQ=10×S/N

Calibration, Limit of Detection, and Limit of Quantitation

Sample analysis of kombucha showed the presence of various organic acids, with gluconic, lactic, acetic, pyruvic, succinic, malic, oxalic, and citric acids as the eight major organic acids. A calibration curve with ten concentration levels ranging from 0.2 mg/L to 150 mg/L was constructed for gluconate. Calibration curves with seven concentration levels ranging from 1 mg/L to 100 mg/L were constructed for lactate, pyruvate, succinate, malate, and citrate, and from 0.2 mg/L to 20 mg/L for oxalate. A calibration curve with eight concentration levels ranging from 5 mg/L to 400 mg/L was constructed for acetate owing to its high concentration in kombucha samples.

Sample Analysis

In Figure 2, Chromatograms A, B, and C show the analysis of three brands of kombucha. Although all the samples are sold as kombucha tea, there are differences in their organic acid composition. The various organic acids were identified by comparing their retention times with those of standards. Acetic acid bacteria from kombucha produce acetic acid as one of the main metabolites, when sucrose is used as a carbon source. It is the predominant organic acid found in kombucha. Gluconic acid is also one of the major organic acids produced as a result of the kombucha fermentation process on the traditional substrate, a black or green tea extract sweetened with 5–8% sucrose. Acetic acid produces an astringent and acidic flavor, while the flavor produced by gluconic acid is mild.

Lactic acid, succinic acid, malic acid, oxalic acid, and citric acid are not characteristic compounds for kombucha tea, but they are found in the three kombucha samples. Pyruvic acid is present in Kombucha B, but is not found in Kombuchas A and C. Propanoic acid is only found in Kombucha A.

It is often claimed that kombucha contains glucuronic acid, a potent detoxifying compound produced by the liver. In reality, no credible laboratory analysis of kombucha tea has found glucuronic acid. In our study, no glucuronic acid was detected.

The concentrations of all the anions in Figure 2 were estimated using the 20-anion standard mixture, except that gluconate, lactate, acetate, pyruvate, succinate, malate, oxalate, and citrate were accurately quantified from their respective calibration curves.

Precision

The precision of an analytical procedure is usually expressed as the relative standard deviation (RSD) of a series of measurements. For our method, the peak area and retention time precision was determined for six replicate injections of a standard mixture containing 0.2 mg/L each of acetate, succinate, and citrate. The retention time RSDs and the peak area RSDs of the three representative analytes are within 0.1% and 7% respectively (Table 3), indicating good method precision at these analyte concentrations.

Table 3. Method precision.

Analyte	RT (min)	RT RSD	Area (nC*min)	Peak Area RSD
Acetate	10.14	0.10	5.46	1.87
Succinate	30.27	0.01	0.03	7.19
Citrate	42.27	0.01	0.03	1.13

Accuracy

The accuracy of our method on the Dionex IonPac AS11-HC-4µm column was verified by determining recoveries of acetate, succinate, and citrate in spiked kombucha samples (Table 4). Samples were spiked at a series of percentages (20, 50, 100, or 150) of the amount measured using standard solutions. Recoveries were calculated from the difference in response between the spiked and unspiked samples. The average recovery for three organic acids ranged from 81 to 115%, indicating that this method can accurately determine organic acids in kombucha samples.

Table 4. Recovery of acetate, succinate, and citrate.

Kombucha A (diluted 20 fold)			
	Acetate (mg/L)	Succinate (mg/L)	Citrate (mg/L)
*Endogenous	136.4	1.03	16.0
Spiked			
20% addition	27	0.3	3
50% addition	70	0.7	8
100% addition	130	1.4	16
150% addition	n.a.	2.1	24
Measured			
20% addition	23.6	0.326	3.03
50% addition	68.5	0.683	8.23
100% addition	131.0	1.36	16.0
150% addition	n.a.	2.16	23.2
Recovery (%)			
20% addition	87	109	101
50% addition	99	98	103
100% addition	101	97	100
150% addition	n.a.	103	97
Kombucha B (diluted 20 fold)			
	Acetate (mg/L)	Succinate (mg/L)	Citrate (mg/L)
*Endogenous	39.0	12.8	3.85
Spiked			
20% addition	8	2	0.8
50% addition	25	5	2
100% addition	60	10	4
150% addition	n.a.	15	6
Measured			
20% addition	9.19	1.90	0.860
50% addition	27.2	4.85	2.19
100% addition	62.7	9.16	4.32
150% addition	n.a.	15.8	6.36
Recovery (%)			
20% addition	115	95	107
50% addition	109	97	109
100% addition	104	92	108
150% addition	n.a	105	106

Kombucha C (diluted 20 fold)			
	Acetate (mg/L)	Succinate (mg/L)	Citrate (mg/L)
*Endogenous	168	2.09	1.65
Spiked			
20% addition	35	0.5	0.32
50% addition	90	1.25	0.8
100% addition	170	2.5	1.6
150% addition	n.a	3.75	2.4
Measured			
20% addition	28.3	0.458	0.295
50% addition	84.2	1.26	0.856
100% addition	169	2.43	1.76
150% addition	n.a	3.62	2.63
Recovery (%)			
20% addition	81	92	92
50% addition	94	101	107
100% addition	99	97	110
150% addition	n.a	96	109

*The endogenous concentration represents the concentration in the 20-fold diluted solution.

Conclusion

This study presents profiles of the inorganic anions and organic acids together with quantitative determination of organic acids in kombucha samples. The method uses a Dionex IonPac AS11-HC-4µm column, which is ideal for separating a wide range of organic acids and inorganic anions present in complex samples, such as kombucha tea, in combination with a HPIC system. The suppressed conductivity detection offers high sensitivity for the anions, including various organic acids. The specificity and sensitivity of this method allow simple sample treatments without complex procedures such as extraction and/or derivatization. In addition, the recovery study shows good method accuracy.

REFERENCES

1. Vijayaraghavan R.; Singh M.; Rao P.V.L.; Bhattacharya R.; Kumar P.; Sugendran K.; Kumar O.; Pant S.C.; Singh R. Subacute (90 days) oral toxicity studies of Kombucha Tea. *Biomed. Environ. Sci.* **2000**, 13, 293–299.
2. Chen C.; Liu B.Y. Changes in major components of tea fungus metabolites during prolonged fermentation. *J. Appl. Microbiol.* **2000**, *89*, 834–839.
3. Jayabalan R.; Marimuthu S.; Swaminathan K. Changes in content of organic acids and tea polyphenols during kombucha tea fermentation. *Food Chem.* **2007**, *102*, 392–398.
4. Thermo Fisher Scientific. Product Manual for Eluent Generator Cartridges. P/N 065018, Sunnyvale, CA, 2012.
5. Thermo Fisher Scientific. Product Manual for Continuously Regenerated Trap Columns (CR-TC). Doc. No 031910, Sunnyvale, CA, 2010.
6. Thermo Fisher Scientific. Product Manual for Dionex ERS 500 suppressor. Doc No. 031956, Sunnyvale, CA, 2013.
7. Brinkmann T.; Specht C.H.; Frimmel F.H. Non-Linear Calibration Functions in Ion Chromatography with Suppressed Conductivity Detection Using Hydroxide Eluents. *J. Chromatogr. A* **2002**, *957*, 99–109.