Application Note 1152

Determination of Carbohydrates in Kombucha Using HPAE-PAD

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Key Words
Glucose, Fructose, Sucrose, Dionex CarboPac PA20 Column, Monosaccharide, Beverage, Disaccharide

Goal
To develop an accurate method for determining simple carbohydrates in kombucha using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD)

Introduction
Kombucha is any of a variety of fermented, lightly effervescent, sweetened black or green tea drinks that are commonly intended as functional beverages. Kombucha 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. Kombucha is said to help regulate blood sugar and possibly help with high blood pressure and high cholesterol, making it a drink of interest to diabetics. The question remains, however, of whether kombucha delivers its reported health benefits.

Sucrose is the most common carbon source in kombucha fermentation. During fermentation, it is believed that most of the sugar is consumed by the bacteria and yeast, so it has minimal effect on blood sugar levels in the body. Generally, sucrose is biochemically converted into fructose and glucose, which are metabolized to gluconic acid and acetic acid. These metabolites are present in the drink.[1] Though most of the sugar is converted into other components during the fermentation process, some remains in the finished tea.

Several methods have been used to determine carbohydrates in kombucha, including an enzymatic method, HPLC, etc.[2,3] HPAE-PAD is a carbohydrate analysis technique that allows the determination of carbohydrates by direct detection, i.e. no sample derivatization is required. HPAE-PAD has high sensitivity for carbohydrates. Therefore, despite the consumption of much of the sucrose during the fermentation, sugar concentrations of some kombucha samples may require several-hundred-fold dilution prior to analysis. Large sample dilutions are a possible source of error.

In this study, a single hardware modification was made to handle high-concentration samples and minimize the amount of sample dilution:

- An increase in the thickness of the spacer gasket to 15 mil (from 2 mil included in the standard package of Gold on PTFE Disposable Electrode) in the electrochemical flow cell to reduce detection sensitivity (P/N 057364)

This study presents a simple, selective, and accurate method, using a Thermo Scientific™ Dionex™ CarboPac™ PA20 column, to determine carbohydrates in kombucha.

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Equipment

- Thermo Scientific™ Dionex™ ICS-5000⁺ system*, including:
  - Gradient or Isocratic Pump, with the vacuum degas option (P/N 063353) installed
  - Thermo Scientific™ Dionex™ EG Eluent Generator module
  - Dionex ICS-5000⁺ DC Compartment
  - Thermo Scientific™ Dionex™ AS-AP Autosampler
  - ED Electrochemical Detector (P/N 079831)
  - Electrochemical Cell (P/N 061757)
  - Gold on PTFE Disposable Electrode (P/N 066480)
  - Gasket, (HDPE) for Disposable Electrodes 0.015" (P/N 057364)
  - pH, Ag/AgCl Reference Electrode (P/N 061879)
  - Thermo Scientific™ Dionex™ EGC III KOH Potassium Hydroxide Eluent Generator Cartridge (P/N 074532)
  - CR-ATC 500 Continuously Regenerated Anion Trap Column (P/N 075550)
  - 10 µL PEEK Sample Loop (P/N 042949)
- 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)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software version 7.2

*Note: This application can also be run on a Thermo Scientific™ Dionex™ Integrion™ HPIC™ system with electrochemical detection.

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

- D-Fructose (Fisher Scientific P/N L96-500)
- Sucrose (Fisher Scientific P/N S5-500)
- D-Glucose (Fisher Scientific P/N D16-1)

Samples

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

Conditions	
Column:	Thermo Scientific Dionex CarboPac PA20 analytical column (3 × 150 mm)
Eluent Source:	Thermo Scientific Dionex EGC III KOH with CR-ATC 500
Eluent:	Potassium hydroxide
Isocratic:	100 mM KOH from -15 min to -10.05 min only for column wash, 10 mM KOH from -10.00 min to 0 min for reequilibration, 10 mM KOH from 0–15 min
Flow Rate:	0.5 mL/min
Injection Volume:	10 µL
Temperature:	30 °C (column and detector compartments)
Backpressure:	~2700 psi
Detection:	Pulsed amperometric
Background:	~40 nC
Working Electrode:	Disposable Au on PTFE electrode
Electrochemical Cell Gasket:	15 mil*
Reference Electrode:	pH, Ag/AgCl, Ag mode
Noise:	30–60 pC

*1 mil = 0.001 inches

Carbohydrate Waveform

Carbohydrate 4-Potential Waveform for the ED

Time (s)	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	–2.0	Off	On	Off
0.42	–2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	–0.1	Off	On	Off
0.50	–0.1	Off	On	Off

*Settings required in the Dionex ICS-3000/5000, but not used in older Dionex IC systems.

Preparation of Solutions and Reagents

Eluent Solutions

Generate the potassium hydroxide (KOH) eluent on line by pumping high-quality degassed DI water through the Dionex EGC III KOH cartridge. The Chromeleon CDS software will track the amount of KOH used and calculate the remaining lifetime.

If desired, the method can be executed with manually prepared eluents—NaOH is used in place of KOH. Add 5.2 mL of 50% w/w NaOH to 994.8 mL of degassed DI water to prepare 1 L of 100 mM NaOH. Proportion the 100 mM hydroxide solution with DI water to produce the listed hydroxide concentrations. See Thermo Scientific Technical Note 71 for detailed information on manual eluent preparation.[4]

Stock Standard Solutions

Dissolve solid standards in DI water to prepare a 1 g/L stock solution for each carbohydrate. Maintain the stock solution at –20 °C until needed.

Working Standard Solutions

Prepare the mixed carbohydrate working standards by diluting the stock solutions as required. Store working standards at 4 °C. All dilutions are made gravimetrically to ensure high accuracy. The concentrations used for glucose calibration were in the range of 0.2–50 mg/L (0.2, 0.5, 1.5, 4.5, 12, 25 and 50 mg/L), for sucrose the calibration range was 10–100 mg/L (10, 12.5, 20, 25, 35, 50 and 100 mg/L) and for fructose the calibration range was 0.2–100 mg/L (0.2, 0.5, 1.5, 5, 16, 50, 100 mg/L).

Sample Preparation

Centrifuge kombucha samples at 6500–7500 g for 15 min; then pass the supernatant through a Nalgene syringe filter (0.2 µm) and dilute 1:500 with DI water prior to analysis.

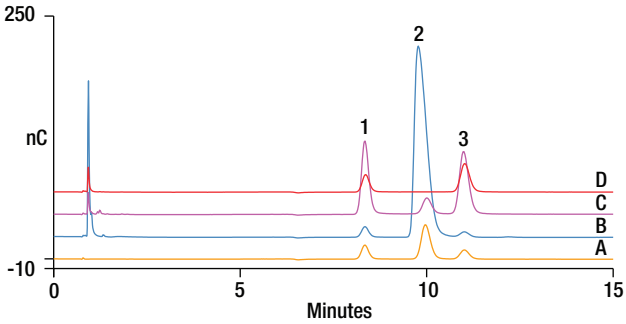
Precautions

Carbohydrates have limited stability unless sterility is maintained. Store solutions and samples at -40 °C. Avoid multiple freeze/thaw cycles to preserve the carbohydrates. Ensure that all carbohydrate solutions are well mixed after thawing stock standards and prior to preparing working standards and spiking solutions. When using the Dionex ICS-5000 EG Eluent Generator module, install the vacuum degas conversion kit (P/N 063353). This degasser will remove gases generated by the Dionex EG Eluent Generation module and help maintain a stable baseline. This kit is not necessary when preparing eluents manually. Keep the eluent water blanketed under 34–55 kPa (5–8 psi) of nitrogen at all times to reduce carbonate contamination and opportunistic microorganisms. When using an older Dionex EG Eluent Generation module, a Dionex CarboPac PA20 guard column should not be installed because there is potential for the system pressure to exceed 3000 psi, which would damage the Dionex EG module degasser (Note that a guard column was not used for this application). Replace the reference electrode every six months and replace the disposable working electrode every four weeks to reduce the probability of failure during analysis.

Results and Discussion

Separation

Figure 1, Chromatogram A, shows the separation of three carbohydrates on a Dionex CarboPac PA20 column. Using hydroxide isocratic elution, glucose, sucrose, and fructose are fully resolved. The total separation time is 15 min with an additional 5 min wash and 10 min equilibration prior to sample injection. The column wash ensures stable retention times during analysis of kombucha samples.



Column:	Dionex CarboPac PA20, Analytical, 3 × 150 mm
Eluent:	100 mM KOH -15–10.05 min, 10 mM -10–0 min, 10 mM 0–15 min
Eluent Source:	Dionex EGC III KOH cartridge with CR-ATC 500
Temperature:	30 °C
Flow Rate:	0.5 mL/min
Inj. Volume:	10 µL
Detection:	PAD, Au on PTFE (Disposable), 15 mil gasket

Peak:
1. Glucose
2. Sucrose
3. Fructose

Figure 1. Separation of glucose, sucrose, and fructose on a Dionex CarboPac PA20 column in (A) a mix of standards, glucose (4.5 mg/L), sucrose (25 mg/L), and fructose (5 mg/L), (B) Kombucha A with 100-fold dilution, (C) Kombucha B with 500-fold dilution and (D) Kombucha C with 500-fold dilution.

Linear Range

Initial analyses showed that in the three kombucha samples, glucose concentration ranged from 0.27 to 12 g/L, sucrose concentration ranged from zero to 2.2 g/L, and fructose concentration ranged from 0.32–1.8 g/L. Samples were subsequently diluted such that the analyte concentrations fell within the linear range. Linearity for three sugars was determined by injecting calibration standards in triplicate, glucose ranging from 0.2–50 mg/L, sucrose from 10–100 mg/L, and fructose from 0.2–100 mg/L. Glucose, sucrose, and fructose were linear with the coefficients of determination at 0.9997, 0.9992, and 1.000, respectively (Table 2). A representative calibration plot for sucrose is shown in Figure 2. Compared with the other two reducing sugars (glucose and fructose), a narrower calibration range was chosen for sucrose to quantify its presence in samples. Carbohydrates are weak acids with pK_as above 11. The use of hydroxide-based eluents at high pH promotes ionization of the carbohydrates to their anionic form. Carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of a gold electrode. Carbohydrates, which undergo transport-controlled reactions, produce linear plots over larger concentration ranges than those carbohydrate with reactions under surface control. Sucrose is a non-reducing disaccharide composed of a D-glucose and a D-fructose bonded with a glycosidic linkage. Because these sugars are linked at their anomeric carbons, sucrose cannot convert to an open-chain form with an aldehyde group. This is thought to be a major reason that the detection of sucrose on the Au electrode is primarily under surface control. In contrast, the alcohol and aldehyde groups on D-glucose can be oxidized, so glucose is primarily under transport control. Fructose is under mixed control. This results in glucose and fructose having a larger linear calibration range than sucrose.[5]

Sample Analysis

Figure 1, Chromatograms B, C, and D show the sugars in the kombucha samples. All three target analytes are present in Kombucha A and B. However, only glucose and fructose are found in Kombucha C. Using a thicker gasket at the working electrode, a 500-fold sample dilution is appropriate for simultaneously quantitating these three sugars. When using with the 2 mil gasket, different dilutions are needed in order for the three samples to have all of the sample sugars fall within their linear calibration range. Therefore, using the 15 mil gasket simplifies the method and reduces the amount of dilution needed for some samples. The concentrations of the three sugars in the three samples are presented in Table 1.

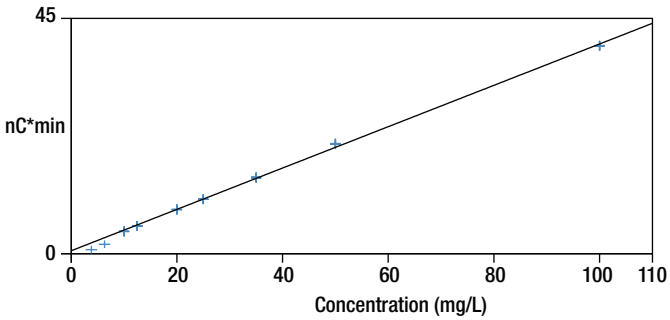


Figure 2. Calibration plot for sucrose in the concentration range of 2.5–100 mg/L. A linear curve in the narrower range (10-100 mg/L) was used for sample analysis.

Table 1. Carbohydrate quantification in kombucha.

Sample	Glucose	Sucrose	Fructose
	g/L (amount present after correcting for the dilution factor)		
Kombucha A	0.266	22.0	0.319
Kombucha B	12.1	5.95	17.8
Kombucha C	2.77	N.A.	7.75

Table 2. Method precisions and calibration.

Analyte	RT (min)	RT RSD	Area (nC*min)	Peak Area RSD	Coefficient of Determination (r^2)
Glucose	8.36	0.04	3.386	0.25	0.9997
Sucrose	9.99	0.00	10.459	0.27	0.9992
Fructose	11.03	0.04	2.791	0.73	1.000

Table 3. Recovery of glucose, sucrose, and fructose.

Kombucha A (Diluted 500 Fold)			
	Glucose (mg/L)	**Sucrose (mg/L)**	**Fructose (mg/L)**
Endogenous	0.53	44.03	0.64
Spiked			
20% addition	0.10	8.00	0.12
50% addition	0.25	20.00	0.30
100% addition	0.50	40.00	0.60
150% addition	0.75	60.00	0.90
Measured			
20% addition	0.098	6.59	0.099
50% addition	0.232	18.1	0.337
100% addition	0.520	35.9	0.645
150% addition (additional 2-fold dilution before injection)	0.600	57.3	0.964
Recovery (%)			
20% addition	98	82	83
50% addition	93	90	112
100% addition	104	90	107
150% addition	80	96	107
Kombucha B (Diluted 500 Fold)			
	Glucose (mg/L)	**Sucrose (mg/L)**	**Fructose (mg/L)**
Endogenous	24.2	11.9	35.5
Spiked			
20% addition	5.00	2.40	7.00
50% addition	12.5	6.00	17.5
100% addition	25.0	12.0	35.0
150% addition	37.5	18.0	52.5
Measured			
20% addition	4.87	3.03	7.32
50% addition	11.8	6.98	17.1
100% addition	22.5	14.2	33.0
150% addition (additional 2- fold dilution before injection)	37.1	20.0	51.4
Recovery (%)			
20% addition	97	126	105
50% addition	94	116	98
100% addition	90	118	94
150% addition	99	111	98
Kombucha C (Diluted 500 Fold)			
	Glucose (mg/L)	**Sucrose (mg/L)**	**Fructose (mg/L)**
Endogenous	5.53		15.5
Spiked			
20% addition	1.00		3.00
50% addition	2.50		7.50
100% addition	5.00		15.0
150% addition	7.50		22.5
Measured			
20% addition	1.02		2.82
50% addition	2.50		7.30
100% addition	4.86		14.6
150% addition	7.56		21.0
Recovery (%)			
20% addition	102		94
50% addition	100		97
100% addition	97		97
150% addition	101		93

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 precisions were determined for six replicate injections of a mixture of sugar standards (Table 2). The concentrations used for precision injections on the Dionex CarboPac PA20 column were 4.5 mg/L (glucose), 25 mg/L (sucrose), and 5 mg/L (fructose). For the kombucha samples, the retention time precisions (RSD) were <0.1% and the peak area precisions ranged from 0.17–4.1%. The high precisions suggest that this method is appropriate for the accurate analysis of these three carbohydrates in kombucha.

Accuracy

The accuracy of our method on the Dionex CarboPac PA20 column was verified by determining recoveries of sugars in spiked kombucha samples (Table 3). Samples were spiked at a series of percentages of the amount measured (20, 50, 100, 150) using standard solutions. Recoveries were calculated from the difference in response between the spiked and unspiked samples. The average recovery for three sugars ranged from 80–126%, indicating that this method can accurately determine carbohydrates in kombucha samples.

Comparison of the 15 mil Gasket to the 2 mil Gasket

The thicker gasket extends the linear calibration range to higher concentrations. Increasing the gasket thickness decreases the linear flow rate at the electrode. Some of the consequences of using a thicker gasket that influence the calibration outcome are:

- Increased coulombic efficiency
- Longer residence time at the electrode
- Lower proportion of analyte reaching the electrode surface by mass transport

Table 4 summarizes the decrease in response of glucose, sucrose, and fructose with the 15 mil gasket relative to the 2 mil gasket. Due to the increased linear range of the method, carbohydrates present in major and minor amounts can generally be measured using a 500-fold dilution instead of the greater dilution (or multiple dilutions) required with the 2 mil gasket.

Table 4. Response with the 15 mil gasket relative to the 2 mil gasket.

Glucose (mg/L)		Sucrose (mg/L)		Fructose (mg/L)	
0.2	30%	10	30%	0.2	49%
0.5	31%			0.5	55%
1.5	35%	20	45%	1.5	60%
4.5	38%			5	62%
12	39%	50	64%	16	65%
25	47%			50	74%

Conclusion

This study describes a method for the accurate, derivatization-free determination of glucose, sucrose, and fructose in kombucha samples. The method uses the Dionex CarboPac PA20 column with electrolytically generated hydroxide eluent and a thicker gasket for the working electrode. The method is shown to have a linear range suitable for handling the wide range of sugar concentrations among the three kombucha brands analyzed with minimal sample treatment.

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