Quantification of stevioside and rebaudioside A in *Stevia rebaudiana* Bertoni leaves using the Agilent 1260 Infinity LC

Application Note

Food Testing & Agriculture

Author

Srividya Kailasam
Agilent Technologies, Inc.
Bangalore, India

Abstract

In this Application Note, we describe the quantification of stevioside and rebaudioside A in *Stevia rebaudiana* Bertoni leaf extracts using the Agilent 1260 Infinity LC. The Agilent ZORBAX carbohydrate column was found to be the most suitable for the separation of the two analytes. The linear dynamic ranges were determined after validating the robustness of critical method parameters. The Agilent 7696A Sample Prep WorkBench was used for the preparation of calibration standards. Both analytes showed good linearity from 1 to 1,000 µg/mL with the R^2 values being > 0.9999. Both the LOD and LOQ values of the two compounds were determined to be 1.0 µg/mL. Good recoveries were obtained for the spiked samples.

Introduction

Diterpene glycosides (stevioside and rebaudioside) extracted from the leaves of *Stevia rebaudiana* Bertoni plants are used in food and beverages as substitutes for synthetic sweeteners. As these compounds are non-nutritive (zero-calorie) and have a high potency (stevioside is 300 times and rebaudioside is 400 times sweeter than sucrose), the leaf extract has been traditionally used in the treatment of diabetes [1, 2]. It is important to characterize the Stevia extract to determine the relative amounts of the various glycosides, which impacts the quality of the product. In a previously published Application Note, the quantification of these two diterpene glycosides using the Agilent ZORBAX carbohydrate column in the range 70 and 700 µg/mL has been demonstrated [3]. In this Application Note, we describe the partial validation and quantification of stevioside and rebaudioside A up to 1,000 µg/mL in *Stevia rebaudiana* Bertoni leaf extract using the Agilent 1260 Infinity LC System.

Agilent Technologies

Method

Instruments and software

The Agilent LC system consisted of the following modules:

- Agilent 1260 Infinity Binary Pump (G1312B)
- Agilent 1260 Infinity Degasser (G1379B)
- Agilent 1260 Infinity Autosampler (G1367E)
- Agilent 1260 Infinity Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316A)
- Agilent 1260 Infinity DAD (G4212B), with Max-Light 60-mm high sensitivity flow cell

Software:	Agilent ChemStation revision B.04.03
Sample preparation:	Agilent 7696A Sample Prep WorkBench

Reagents and materials

Rebaudioside A, stevioside and *Stevia rebaudiana* Bertoni leaves (Sigma), and acetonitrile (Labscan) were used in this study.

Chromatographic parameters

Column	Agilent ZORBAX Carbohydrate Analysis Column 4.6 × 150 mm, 5 µm (p/n 843300-908)
Mobile phases	A: Water 30% B: ACN 70%
Injection volume	5 µL
ALS thermostat	6 °C
Flow rate	1.0 mL/min (isocratic analysis)
Column temperature	30 °C
Detection	205 nm, 4 nm BW; Ref: No; PW > 0.25 s (20 Hz)

Standards

Stock solutions were prepared in a mix of 30% water and 70% acetonitrile. The method was validated using a solution containing 100 µg/mL of each analyte. To prepare calibration curves, solutions containing 0.5, 1.5, 2.5, 5, 10, 25, 50, 100, 250, 500, 1,000 µg/mL of each standard in 30% water and 70% acetonitrile were used.

The Agilent 7696A Sample Prep WorkBench[4] was used serially to dilute the 2000 µg/mL stock solutions of rebaudioside A and stevioside to obtain a series of calibration standards containing 1.0, 2.5, 5, 10, 25, 50, 100, 250, 500, 1,000 µg/mL of each standard in 30% water and 70% acetonitrile mix. Table 1 shows the syringe parameters used in the WorkBench method.

Sample preparation

Stevia rebaudiana Bertoni leaves were crushed and approximately 0.1 g of the powder was weighed into a 20 mL glass vial. Ten milliliters of 30% water and 70% ACN mix was added to the vial which was then vortexed. The extraction was carried out by sonication for 60 minutes. The contents of the vial were centrifuged and the supernatant diluted 10 times with 30% water and 70% ACN mix. To an aliquot of the 10 times diluted sample, we added 2,000 µg/mL stock solutions of rebaudioside and stevioside to test analyte recoveries. The final concentration of each analyte in the spiked sample was 100 µg/mL. Five microliters of the unspiked and spiked sample solutions were injected for analysis.

Results and discussion

Separation

After testing several stationary phases and chromatographic conditions, the Agilent ZORBAX carbohydrate analysis column was found to be the most suitable for the analysis of rebaudioside A and stevioside. A typical chromatogram of the 100 µg/mL calibration standard is shown in Figure 1.

	Solvent prewash 1	Dispense wash	Dispense pump	Dispense setting
Number of washes	1	1	2	-
Wash volume (µL)	100	50	50	-
Draw speed (µL/min)	1,000	200	200	200
Dispense speed (µL/min)	1,000	200	200	200
Needle depth offset (mm)	0	-2	-2	-2
Viscosity delays (s)	-	0	0	0
Turret solvent	A	-	-	-
Air gap (% syringe vol.)	-	-	-	0

Table 1
500 µL syringe parameters.

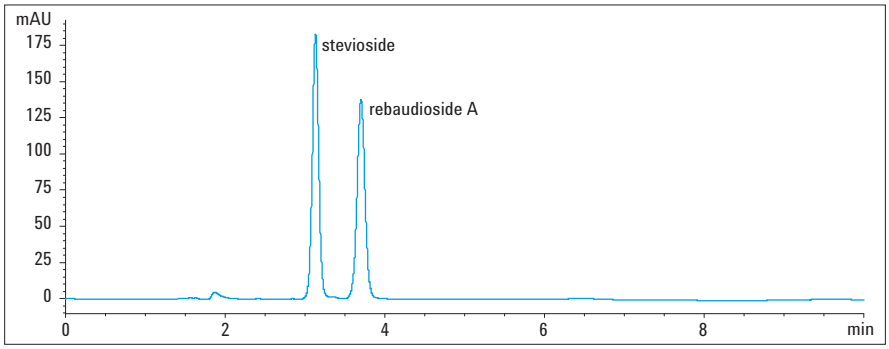

Figure 1
Chromatogram of the 100 µg/mL calibration standard.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ values were determined by dividing the peak heights by peak to peak noise between 0.1–0.4 minutes. It was found that the stevioside and reabudioside A peaks at the 1 µg/mL have signal-to-noise values of 56.2 and 43.7. As no significant peaks were observed for the 0.5 µg/mL level, 1 µg/mL was chosen as the LOD and LOQ for the method.

Linearity

The calibration samples prepared using the Agilent 7696A Sample Prep WorkBench were used to test the method linearity. The area responses plotted against the concentration values were found to be linear between 1.0 and 1,000 µg/mL for both the analytes. The calibration data for stevioside and rebaudioside A are given in Table 2. We observed an improvement in the R^2 values when the calibration standards were prepared using the 7996A Sample Prep WorkBench instead of manual dilution.

Precision of retention times and areas

Calibration samples containing 0.5 to 1,000 µg/mL of both analytes were prepared by manual dilution. Very small peaks that could not be integrated were observed for the 0.5 µg/mL calibration sample. All the other calibration standards were injected 10 times and the last six replicates were used to calculate the RSD values of peak areas and retention times. The RSDs for the retention times of both compounds at all calibration levels were found to be ≤ 0.13%. The peak area RSDs for the two compounds at the various concentration levels were < 4% except for 1.5 and 2.5 µg/mL levels of stevioside at which the RSDs were > 5%.

Robustness

After the preliminary method development, a set of method parameters were systematically varied to test the robustness of the method. As a readout of the impact of parameter variations on the results, we monitored the deviations in retention times and peak areas. Table 3 shows the percentage deviations observed for the retention times and peak areas of the analytes as the parameters were varied. It was observed that the deviations in the retention times were well within the set limit of 3% for all the parameter changes while the deviations in the peak areas were within the set limit of 5% for all the parameters changes except the detection wavelength.

Analyte	R^2	Linear regression
Stevioside	0.99991	Area = 9.86* Amount + 20.18
Rebaudioside	0.99995	Area = 9.50* Amount + 13.30

Table 2
Calibration data for Stevioside and Rebaudioside A standards prepared using the Agilent 7696A Sample Prep WorkBench.

	Stevioside		Rebaudioside A	
Parameter changed	Deviation in the retention time (%)	Deviation in the area (%)	Deviation in the retention time (%)	Deviation in the area (%)
Flow - 2% (0.98 mL/min)	2.51	1.36	2.75	1.79
Flow + 2% (1.02 mL/min)	-2.31	-2.55	-2.48	-2.48
Column temperature - 5% (28.5 °C)	-0.33	0.33	-0.47	-0.22
Column temperature + 5% (31.5 °C)	-1.04	-0.73	-1.5	- 0.96
Injection volume - 5% (4.8 µL)	-0.54	-3.81	-0.93	-3.73
Injection volume + 5% (5.2 µL)	-0.85	4.33	-1.36	3.76
Detection wavelength - 3 nm (202 nm)	-1.16	19.09	-1.78	17.06
Detection wavelength + 3 nm (208 nm)	-1.30	-31.33	-2.01	-31.40

Table 3
Method robustness: Effect of method parameter changes on the retention times and peak areas.

Recovery from sample matrix

The overlaid chromatograms of the unspiked and spiked *Stevia rebaudiana* Bertoni leaf extract is shown in Figure 2. Table 4 shows excellent recoveries of the analytes added to the diluted stevia extract.

Conclusions

In this Application Note, we describe the detection and quantification of two diterpene glycosides, stevioside and rebaudioside A, extracted from the leaves of *Stevia rebaudiana* Bertoni plants. The developed method is robust, sensitive and reproducible. The Agilent 7696A Sample Prep WorkBench was used for diluting samples for preparing calibration standards. The peak areas for both the analytes were found to be linear between 1 and 1,000 µg/mL with the R^2 values being greater than 0.9999. Hence the 7696A Sample Prep WorkBench can be used for routine application in a QC environment which will reduce variability caused by manual errors. The recoveries of the two analytes spiked into the diluted stevia extract were found to be excellent.

References

1. N. W. Megeji, J. K. Kumar, Virendra Singh, V. K. Kaul and P. S. Ahuja, "Introducing Stevia rebaudiana, a natural zero-calorie sweetener", CURRENT SCIENCE, 88 (5), 801-804, **2005**.
2. N. Kolb, J. L. Herrera, D. J. Ferreyra, and R. F. Uliana, "Analysis of Sweet Diterpene Glycosides from Stevia rebaudiana: Improved HPLC Method", J. Agricultural and Food Chem, 49, 4538-4541, **2001**.
3. "Isocratic Stevia Sweetener Analysis using Selective ZORBAX Columns", Agilent Application Note, Publication Number 5990-3933EN, **2010**.
4. Automated Sample Preparation of Headspace Standards Using the Agilent 7696 WorkBench" Agilent Application Note, Publication Number 5990-9025EN, **2011**.

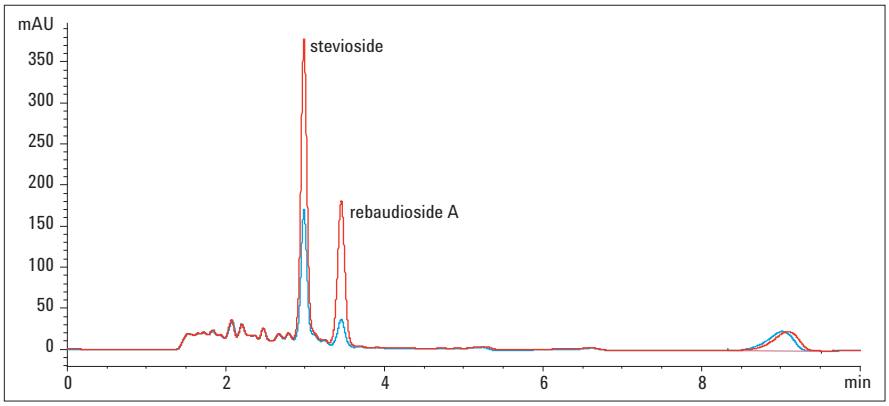

Figure 2
Overlaid chromatograms of the spiked and unspiked Stevia leaf extract.

	Amount in the 10× diluted extract (µg/mL)	Amount spiked during diluting the extract (2nd aliquot) (µg/mL)	Amount found in the diluted and spiked aliquot (µg/mL)	Recoveries
Stevioside	84.30	100	192.83	108.53%
Rebaudioside	21.56	100	114.57	93.01%

Table 4
Calculated concentrations and recoveries in unspiked and spiked diluted stevia extract samples

www.agilent.com/chem

Published in USA, December 1, 2011
Publication Number 5990-9524EN

Agilent Technologies