Application Note: 293

Steviol Glycoside Determination by HPLC with Charged Aerosol and UV Detections Using the Acclaim Trinity P1 Column

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Key Words

- Stevia
- Rebaudioside A
- HILIC
- Natural Sweeteners

Introduction

In December 2008, the U.S. FDA recognized rebaudioside A purified from *Stevia rebaudiana* (Bertoni) as Generally Recognized as Safe (GRAS) for use as a sugar substitute in foods.[1,2] Since this recognition, stevia products have become popular as table-top and beverage sweeteners. Although the stevia plant and extracts from stevia leaves have long been used as sweeteners in Asia and Latin America, the terpene glycosides have different flavor profiles with both sweet and unpleasant bitter flavors.[3] Two steviol glycosides, stevioside and rebaudioside A, are largely responsible for the desired sweet flavor of the leaves (Figure 1), with rebaudioside A preferred for sweeteners.[4]

Steviol glycoside determination is challenging for multiple reasons. The structures of the steviol glycosides are quite similar, differing in small changes in glycosylation. For example, rebaudioside B, an impurity that can be formed during processing of the leaves, differs in structure from rebaudioside A primarily by the presence or absence of a glucose residue at the R1 position on the terpene (Figure 2). These structural similarities make chromatographic separation difficult. In addition to the separation challenges, sensitive detection of these compounds also can be difficult. They do not absorb strongly in the UV, and typical detection wavelengths for steviol glycosides, such as 210 nm, are nonspecific. Other detection methods, such as charged aerosol, can be used in addition to UV detection to improve steviol glycoside quantification.

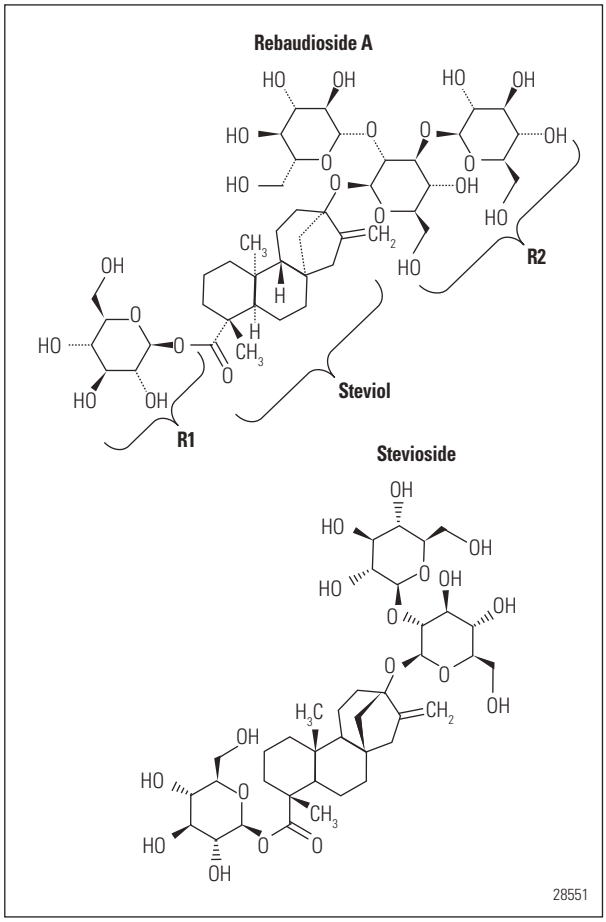

Figure 1: Chemical structures of the steviol glycosides stevioside and rebaudioside A.

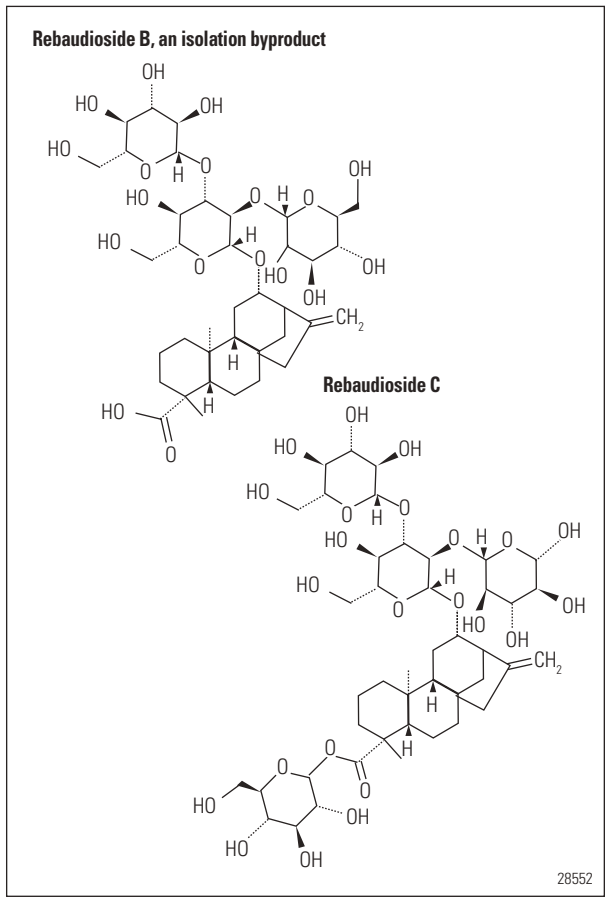

Figure 2: Chemical structures of rebaudioside B and rebaudioside C.

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Typically, individual steviol glycosides are determined by liquid chromatography methods using a polar amine column. The Joint FAO/WHO Commission on Food Additives (JECFA) has published a monograph for the determination of steviol glycosides by UV detection at 210 nm using 80/20 acetonitrile/water at pH = 3.0, adjusted with phosphoric acid.[5] This method specifies a 3.9–4.6 mm i.d. format column and can use flow rates of up to 2.0 mL/min to meet a retention time requirement of 10 min for stevioside. This high flow rate, coupled with a 25 min run time, results in 25–50 mL of mobile phase used per sample. The U.S. Pharmacopeia (USP) rebaudioside A monograph in the Food Codex is similar to the JECFA monograph. It uses an acetate buffer to adjust the pH rather than phosphoric acid, and a propylamine silane phase bonded to a silica gel column.[6] This method uses UV detection and a flow rate of 1.5 mL/min with a run time of up to 70 min, thereby requiring 105 mL of mobile phase per sample.

Here, steviol glycosides are determined by charged aerosol and UV detections in consumer sweeteners. The glycosides are separated on the Thermo Scientific Acclaim Trinity P1 column using 81/19 acetonitrile/ammonium formate buffer at pH = 3.0. This method uses the hydrophilic interaction liquid chromatography (HILIC) mode of the trimode column allowing separation of multiple steviol glycosides. By using a volatile mobile phase, charged aerosol detection is feasible, which adds further flexibility to the method for detection of glycosides that do not have strong UV extinction coefficients. Furthermore, the 2.1 mm i.d. column format reduces solvent use to 3–9 mL/sample compared to the range of 25–105 mL/sample for the JECFA and USP methods.

Equipment

Thermo Scientific Dionex UltiMate Rapid Separation LC (RSLC) system including:

- SRD-3600 Integrated Solvent and Degasser (P/N 5035.9230)
- HPG-3400RS Binary Pump with Solvent Selector Valves (P/N 5040.0046)
- WPS-3000TRS Analytical Autosampler (P/N 5840.0020)
- TCC-3000RS Thermostatted Column Compartment (P/N 5730.0000)
- DAD-3000RS Diode Array Detector (P/N 5082.0020)
- Thermo Scientific Dionex Corona *ultra* Charged Aerosol Detector (P/N 70-9298)

Polypropylene injection vials with caps and septa, 300 µL (Thermo Scientific Dionex P/N 055428)

Nalgene™ Filter Unit, 0.2 µm nylon membrane, 1 L capacity (Thermo Scientific Nalgene P/N 164-0020)

Reagents and Standards

Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better

pH buffer, 4.00 (VWR P/N BDH4018-500ML)

pH buffer, 2.00 (VWR P/N BDH5010-500ML)

Stevia Standards Kit, (ChromaDex P/N KIT-00019566-005) containing:

- Rebaudioside A
- Stevioside
- Rebaudioside B
- Rebaudioside C
- Dulcoside A
- Steviolbioside
- Rebaudioside D

Formic Acid (Sigma-Aldrich P/N 06440)

Ammonium Formate (Sigma-Aldrich P/N 51691)

Acetonitrile (Honeywell P/N 015-4)

Samples

Brand A: sweetener containing stevia leaf extract and inulin

Brand B: sweetener containing rebaudioside A and erythritol

Conditions

Column:	Acclaim™ Trinity™ P1 (3 µm), 2.1 × 100 mm (P/N 071389) Acclaim Trinity P1 (3 µm), 2.1 × 10 mm guard column (P/N 071391) with Guard Holder (P/N 069580)
Mobile Phase:	81/19 acetonitrile/10 mM ammonium formate, pH = 3.0
Flow Rate:	0.3 mL/min
Inj. Volume:	5 µL
Temperature:	20 °C
Detection:	Diode Array UV-vis detector, 210 nm Charged aerosol detector, nebulizer temperature, 10 °C
System Backpressure:	~1500 psi
Noise:	~0.15 mAU (UV) ~0.07 pA (charged aerosol)
Run Time:	10 or 30 min

Preparation of Solutions and Reagents

Mobile Phase Preparation

Transfer 0.63 g of ammonium formate to a 1 L bottle and add 1000 g (1000 mL) of DI water. Adjust the pH of the resulting 10 mM ammonium formate solution to 3.00 ± 0.05 by adding 1700 µL of formic acid. Using a precleaned (with DI water) 0.2 µm nylon filter unit, filter the stock buffer to remove insoluble particles.

Transfer 192.5 mL (192.5 g) 10 mM ammonium formate solution to a 1 L volumetric glass flask and add acetonitrile to fill the flask to the mark. The resulting solution will be approximately 644 g acetonitrile. Mix well. After converting the mass of acetonitrile to a volume, based on density, this method prepares a solution of 81/19 (v/v) acetonitrile/ammonium formate. Mixing aqueous ammonium formate and acetonitrile is endothermic and the solution will cool, resulting in a substantial reduction in volume. This volume change may cause variability in the actual mobile phase composition. These changes in the mobile phase composition will change analyte retention times, and for this reason, gravimetric preparation of the mobile phase will provide the most consistent retention times between mobile phase preparations. Allow the solution to return to ambient temperature before use.

Standards and Sample Solutions

Standards

Prepare individual 2.0 mg/mL steviol glycoside stock standards as shown in Table 1. Additionally, prepare a retention time standard of rebaudioside C by adding approximately 0.5 mg to 500 µL of mobile phase. Combine 50 µL of each solution (dulcoside A, stevioside, rebaudioside A, rebaudioside B, steviolbioside, and rebaudioside C) for a total of 350 µL of solution. Use this combined stock standard of 0.280 mg/mL steviol glycosides to prepare standards of 0.007 mg/mL to 0.190 mg/mL of dulcoside A, stevioside, rebaudioside A, steviolbioside, and rebaudioside B.

Table 1: Stock standards preparation.

Analyte	Amount (mg)	Volume Mobile Phase (µL)	Stock Concentration (mg/mL)
Dulcoside A	1.1	550	2.0
Stevioside	1.8	900	2.0
Rebaudioside A	1.6	800	2.0
Steviolbioside	1.4	700	2.0
Rebaudioside B	1.0	500	2.0

Samples

Prepare samples for analysis by extracting 0.10 g of sweetener with 10 mL of mobile phase. Brand A contains inulin, which is minimally soluble in organic solvents. Vortex the samples for a minimum of 20 s at least four times to dissolve the glycosides. Remove insoluble inulin by filtration through a 0.2 µm Acrodisc® IC syringe filter. Fully dissolve Brand B. Due to the higher concentrations of steviol glycosides in Brand A samples, dilute them fourfold with mobile phase before injection.

Precautions

Take care to consistently prepare the mobile phase. Changes in the ionic strength, pH, or organic content of the mobile phase can lead to shifts in analyte retention times. If chromatographic resolution decreases without a change in overall peak shape, reprepare the ammonium formate buffer, paying close attention to the amount of ammonium formate and the final pH. Increasing the amount of acetonitrile by up to 5% in the mobile phase will increase retention times, which may improve the resolution for complex samples; however the late-eluting peak sensitivity will decrease due to peak broadening from dispersion during the isocratic elution.

Metal contamination of the column will reduce both column efficiency and capacity. If reduced retention times coupled with poor peak shape are observed, remove the Corona™ *ultra*™ Charged Aerosol Detector from the flow path and follow the column wash procedure in Section 4 of the Acclaim Trinity P1 column manual.[7] Be sure to thoroughly equilibrate the column with the ammonium formate mobile phase before reconnecting the Corona *ultra* Charged Aerosol Detector.

For this work, a column temperature of 20 °C was chosen to maximize resolution between dulcoside A and components within stevia leaf extracts. Temperatures between 15–35 °C were evaluated. For the mobile phase conditions, 20 °C was determined to provide the best separation between the early eluting components. For samples which have been purified and do not contain raw leaf extracts, column temperatures between 20–30 °C may be used; however, a temperature-controlled column compartment is recommended to ensure consistent retention times of all components within a sample.

Table 2: Calibration (0.007–0.28 mg/mL for each steviol glycoside) and precision (n = 7) of standard injections. Precision values calculated for 7 injections of a 70 µg/mL standard of each of the glycosides.

Analyte	Detector	RT (min)	RT RSD (%)	Peak Area (mAU*min) or (pA*min)	Peak Area RSD (%)	Coeff. of Deter.	Calibration Model
Dulcoside A	UV: 210 nm	3.40	0.08	2.78	1.40	0.9997	linear
Dulcoside A	Charged Aerosol	3.44	0.25	1.10	1.03	0.9983	quadratic
Stevioside	UV: 210 nm	3.51	0.11	5.28	0.47	0.9996	linear
Stevioside	Charged Aerosol	3.54	0.24	2.15	0.70	0.9969	quadratic
Rebaudioside A	UV: 210 nm	4.42	0.14	2.90	0.58	0.9995	linear
Rebaudioside A	Charged Aerosol	4.46	0.18	1.62	0.53	0.9984	quadratic
Steviolbioside	UV: 210 nm	5.38	0.07	4.24	0.86	0.9996	linear
Steviolbioside	Charged Aerosol	5.42	0.15	1.70	0.35	0.9990	quadratic
Rebaudioside B	UV: 210 nm	6.30	0.09	4.62	0.87	0.9995	linear
Rebaudioside B	Charged Aerosol	6.33	0.21	2.30	0.75	0.9987	quadratic

Results and Discussion

Separation

Figure 3 shows the separation of steviol glycosides on the Acclaim Trinity P1 column. In Figure 3A, stevioside and rebaudioside A are easily observed by UV detection. In UV and charged aerosol detections, the rebaudioside A peak is well resolved with no interferences. Stevioside may closely elute with dulcoside A; however, the two peaks are clearly identifiable. Mogroside V was also added to the standard mixture, but is discussed separately.[8]

Quantification Assay Linearity, LOD, and LOQ

Table 2 shows the linearity for several steviol glycosides determined using UV detection. As shown, the coefficients of determination for UV detection are 0.9996 and 0.9995 for rebaudioside A and stevioside, respectively. The coefficients of determination for rebaudioside A and stevioside when detected by charged aerosol detection were 0.9984 and 0.9969, respectively. Calibration curves using charged aerosol detection are inherently nonlinear and were fit with quadratic curves. This nonlinearity is the result of physical interactions that contribute to charged aerosol detection. To fit the calibration curves for charged aerosol detection, use the quadratic fitting option within the Thermo Scientific Dionex Chromeleon Chromatography Data System software. Coefficient of determination values reported within Chromeleon™ software are from linear fits of converted data. These values are reported in Table 2 for each of the steviol glycosides investigated.

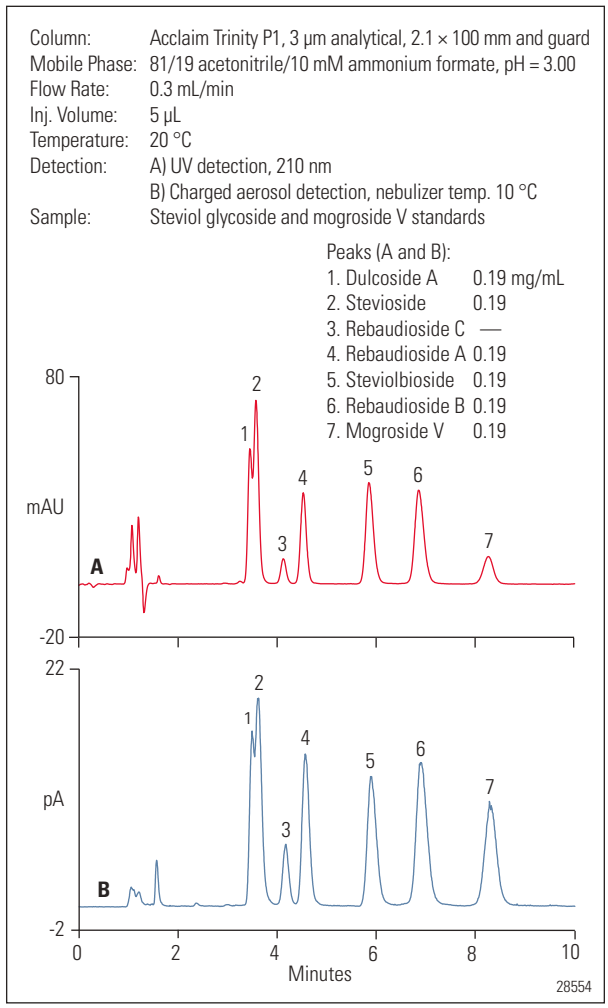

Figure 3: Separation of steviol glycoside standards on the Acclaim Trinity P1 column.

The limits of quantification (LOQs) and limits of detection (LODs) for the steviol glycosides are different for the two detection methods, and charged aerosol detection detects the steviol glycosides with greater sensitivity than UV at 210 nm (Table 3). However, the improvement in sensitivity varies by the individual compound. For example, the LOQ for rebaudioside A is improved by a factor of 3 from 7.0 µg/mL to 2.3 µg/mL by using charged aerosol detection compared to UV at 210 nm. For comparison, the sensitivity increases a factor of 2, from 2.3 µg/mL to 4.6 µg/mL, for steviolbioside.

Table 3: LOQ and LOD based on injections of standards.

	LOQ (µg/mL)		LOD (µg/mL)	
Analyte	**UV: 210 nm**	**Charged Aerosol**	**UV: 210 nm**	**Charged Aerosol**
Dulcoside A	4.6	2.3	1.4	0.7
Stevioside	4.5	1.4	1.2	0.4
Rebaudioside A	7.0	2.3	2.3	0.7
Steviolbioside	4.6	2.3	1.4	0.7
Rebaudioside B	7.0	2.3	2.3	0.7

Sample Analysis

Figure 4 shows the separation of a sweetener extract that is derived from extracted stevia leaves and dispersed in inulin. There are several steviol glycosides detected in this product. When analyzed by UV, which is standard by the JECFA and USP monographs, three steviol glycosides are easily determined. Stevioside, rebaudioside C, and rebaudioside A are each present and baseline resolved. When the sample is analyzed using charged aerosol detection, there are a number of additional peaks that are not observed by UV (Figure 4B). The early eluting unlabeled peaks are from unglycosylated terpenes. Rebaudioside A is resolved from other peaks, stevioside is separated from inulin present in this sample, and a small amount of steviobioside that is not obvious by UV is detected by charged aerosol detection. Quantification of stevioside and rebaudioside A is equivalent by both detection methods. Figure 5 shows an extended chromatogram of the sample in Figure 4. Because the Corona *ultra* Charged Aerosol Detector is a near-universal detector, the presence of sodium in a sample, which is likely in natural products, can be detected. Under these conditions, sodium elutes at 20.4 min and the run time must be extended to avoid coelution of the sodium with analyte peaks in following injections.

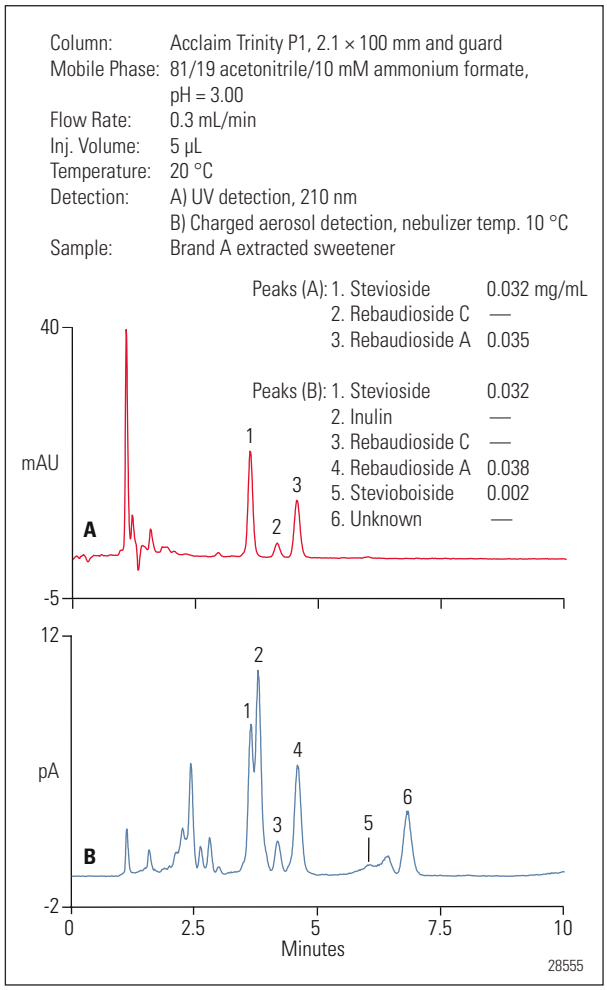

Figure 4: Separation of Brand A sweetener on the Acclaim Trinity P1 column and detected by A) UV and B) charged aerosol detections.

Column:	Acclaim Trinity P1, 3 µm analytical 2.1 × 100 mm and guard
Mobile Phase:	81/19 acetonitrile/10 mM ammonium formate, pH = 3.00
Flow Rate:	0.3 mL/min
Inj. Volume:	5 µL
Temperature:	20 °C
Detection:	Charged aerosol detection, nebulizer temp. 10 °C
Sample:	A) Brand A extracted sweetener B) Steviol glycoside standards

Peaks (A):	1. Stevioside	0.028 mg/mL
	2. Inulin	—
	3. Rebaudioside C	—
	4. Rebaudioside A	0.038
	5. Steviolbioside	0.002
	6. Unknown	—
	7. Sodium	—
Peaks (B):	1. Dulcoside A	0.040 mg/mL
	2. Stevioside	0.040
	3. Rebaudioside C	—
	4. Rebaudioside A	0.040
	5. Steviolbioside	0.040
	6. Rebaudioside B	0.040
	7. Mogroside V	0.040
	8. Sodium	—

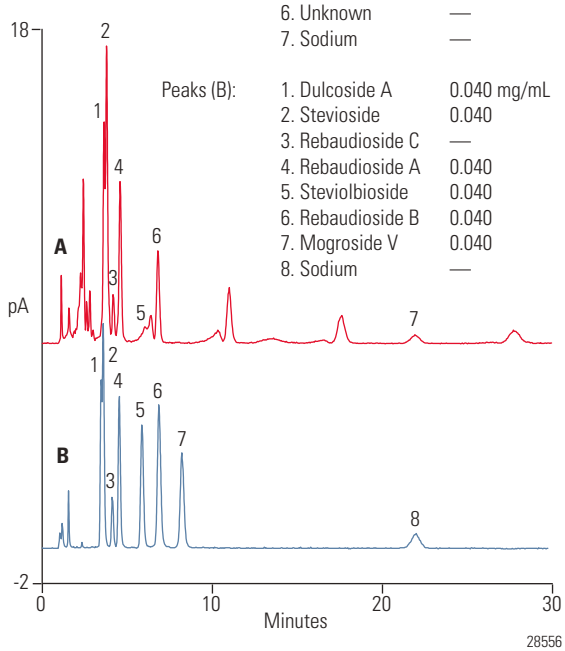

Figure 5: Separation of a A) stevia sweetener Brand A in comparison to B) standards on the Acclaim Trinity P1 column detected by charged aerosol detection.

Figure 6 shows the separation of a 10 mg/mL solution of a commercial table top sweetener composed of rebaudioside A, the FDA approved stevia sweetener. In this sweetener, the rebaudioside A is mixed with erythritol. As shown in Figure 6A, primarily rebaudioside A is observed when analyzing samples using UV detection, with a possible identification of rebaudioside B. When using charged aerosol detection (Figure 6B), erythritol, rebaudioside A, and a small amount of rebaudioside B are observed. The quantification of rebaudioside A is equivalent by both detection techniques.

Column:	Acclaim Trinity P1, 3 µm analytical, 2.1 × 100 mm and guard	
Mobile Phase:	81/19 acetonitrile/10 mM ammonium formate, pH = 3.00	
Flow Rate:	0.3 mL/min	
Inj. Volume:	5 µL	
Temperature:	20 °C	
Detection:	A) UV detection, 210 nm B) Charged aerosol detection	
Sample:	Brand B extracted sweetener	
Peaks (A):	1. Rebaudioside A	0.044 mg/mL
(Inset):	2. Rebaudioside B	<LOD
Peaks (B):	1. Erythritol	—
	2. Rebaudioside A	0.044
	3. Rebaudioside B	0.003
(Inset):	3. Rebaudioside B	0.003

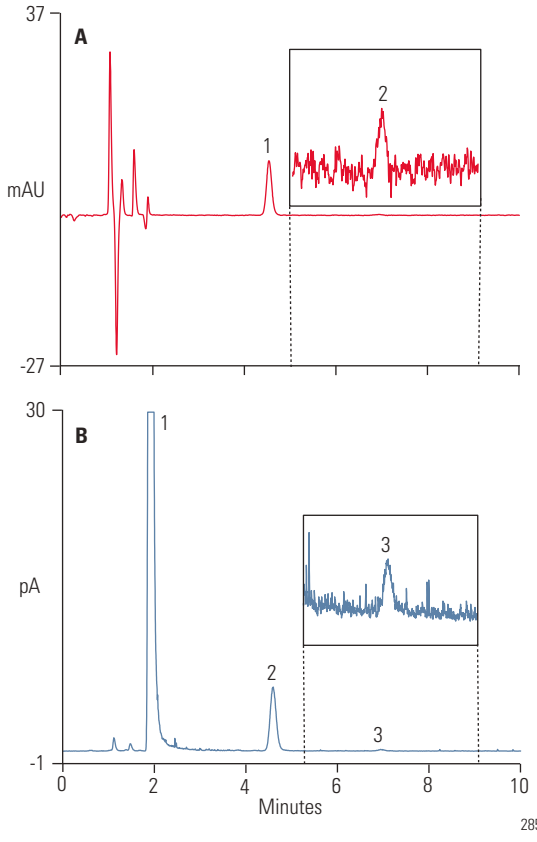

Figure 6: Rebaudioside A determination in Brand B, commercial sweetener, by A) UV and B) charged aerosol detection.

Precision and Accuracy

In addition to assay linearity, Table 2 also summarizes the standard injection precision data for both UV and charged aerosol detection. Peak area RSDs range from 0.47 to 1.40 with the charged aerosol detection peak area precision similar to the UV peak area precision. Retention time precision is excellent with retention time RSDs ranging from 0.07 to 0.25. Retention time variability between batches of mobile phase was within 1.3% for rebaudioside A. These data indicate that with consistent mobile phase preparation, the method is reproducible.

Table 4: Intra- and between-day precision of sweetener analysis.

Day/Sample	Analyte	Detector	Average mg Analyte/g Sweetener	Intraday Precision (RSD)	Interday Precision (RSD)
Day 1 Brand A	Rebaudioside A	UV	13	5.0	2.3
	Rebaudioside A	Charged Aerosol	15	1.5	4.5
	Stevioside	UV	12	5.1	1.6
	Stevioside	Charged Aerosol	10	6.2	10
Day 2 Brand A	Rebaudioside A	UV	14	4.6	—
	Rebaudioside A	Charged Aerosol	15	4.6	—
	Stevioside	UV	12	4.0	—
	Stevioside	Charged Aerosol	11	1.9	—
Day 3 Brand A	Rebaudioside A	UV	14	5.3	—
	Rebaudioside A	Charged Aerosol	16	11	—
	Stevioside	UV	12	3.1	—
	Stevioside	Charged Aerosol	12	10	—
Day 1 Brand B	Rebaudioside A	UV	3.9	4.4	12
	Rebaudioside A	Charged Aerosol	4.2	5.1	13
Day 2 Brand B	Rebaudioside A	UV	3.6	6.9	—
	Rebaudioside A	Charged Aerosol	3.8	6.7	—
Day 3 Brand B	Rebaudioside A	UV	4.8	7.1	—
	Rebaudioside A	Charged Aerosol	5.1	8.5	—

Table 5: Recovery of standards added to Stevia sweetener samples, n = 3.

	Analyte	Detector	Determined Amount with Spiking (mg/mL)	Amount Spiked (mg/mL)	Recovery (%)
Brand A	Rebaudioside A	UV	0.076	0.051	94
	Rebaudioside A	Charged Aerosol	0.080	0.051	85
	Stevioside	UV	0.076	0.051	98
	Stevioside	Charged Aerosol	0.068	0.051	87
Brand B	Rebaudioside A	UV	0.080	0.051	92
	Rebaudioside A	Charged Aerosol	0.079	0.051	88
	Stevioside	UV	0.053	0.051	104
	Stevioside	Charged Aerosol	0.046	0.051	92

Samples of the two sweeteners were analyzed in triplicate over three days. Table 4 shows the calculated mass of stevioside and rebaudioside A in a 1 g portion of the sweeteners along with intraday and interday precision. The determined concentrations of glycosides in the samples are consistent by both UV and charged aerosol detection. During three days of analysis, Brand B was determined to contain 3.6–4.8 mg/g rebaudioside A by UV and 3.8–5.1 mg/g by charged aerosol detection. This corresponds to the expected amount of ~4 mg/g specified for use as a sweetener.[2]

Brand A contains higher concentrations of steviol glycosides in addition to rebaudioside A than the commercial Brand B sweetener because Brand A is an extract of the stevia leaves without further purification to isolate an individual compound. Table 4 shows the calculated masses of stevioside and rebaudioside A in the sample. As with Brand B, the concentrations determined by UV and charged aerosol detection methods are well correlated. The intraday precision for Brands A and B, which range from 1.5–11% from triplicate sample preparations, are similar to the interday precisions of 1.6–10%.

Accuracy of the method was tested by spiking standards at known concentration into samples of Brand A and Brand B sweeteners (Table 5). Rebaudioside A recoveries range from 85–104% by charged aerosol detection and 82–102% by UV detection at 210 nm. These recoveries are quite comparable and suggest overall accuracy of the method. Similarly, stevoiside recoveries range from 81–98% by charged aerosol detection and 84–105% by UV detection.

Conclusion

Here, steviol glycosides are determined by charged aerosol detection and UV detection in consumer sweeteners. Using the Acclaim Trinity P1 column, the proposed method resolves the steviol glycosides of interest. Only 3–9 mL of mobile phase/sample are required compared to 25–60 mL/sample for the JECFA and USP methods, respectively. The reduction in mobile phase use provides savings in both time and resources during mobile phase preparation and reduces waste. Quantification of the two principal glycosides, stevioside and rebaudioside A, was accomplished by both detection methods. In addition, charged aerosol detection has the added advantage of distinguishing additional components in the sample that are not UV absorbing, allowing for a flexible method to check sweetener purity.

Suppliers

VWR, 1310 Goshen Parkway, West Chester, PA 19380
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U.S.A., Tel: 949-419-0288. www.chromadex.com

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