Determination of Steviol Glycosides by HPLC with UV and ELS Detections

Introduction

The stevia plant and extracts from stevia leaves have long been used as sweeteners in Asia and Latin America. The U.S. Food and Drug Administration (US FDA), however, has not approved stevia leaf for use in food and therefore, stevia extracts in the U.S. have been limited to dietary supplements.[1] The sweet components in the leaf extract, known as steviol glycosides, are closely related in structure and have both sweet and bitter flavor profiles.

Two steviol glycosides present in plant tissue, stevioside and rebaudioside A, are largely responsible for the sweet flavor of stevia leaves (Figure 1). Both compounds are approximately 300 times sweeter than sucrose. Additionally, rebaudioside A exhibits reduced bitterness compared to stevioside when used at low to medium sweetening levels.[2] In December 2008, the US FDA approved the request to grant rebaudioside A (also known as rebiana), purified from stevia rebaudiana (Bertoni), Generally Recognized as Safe (GRAS) status for use as a sugar substitute in foods.[3,4] This recognition allows the use of rebiana as a commercial sweetener. Multiple other combinations of steviol glycosides may also be allowed in the future under GRAS status.

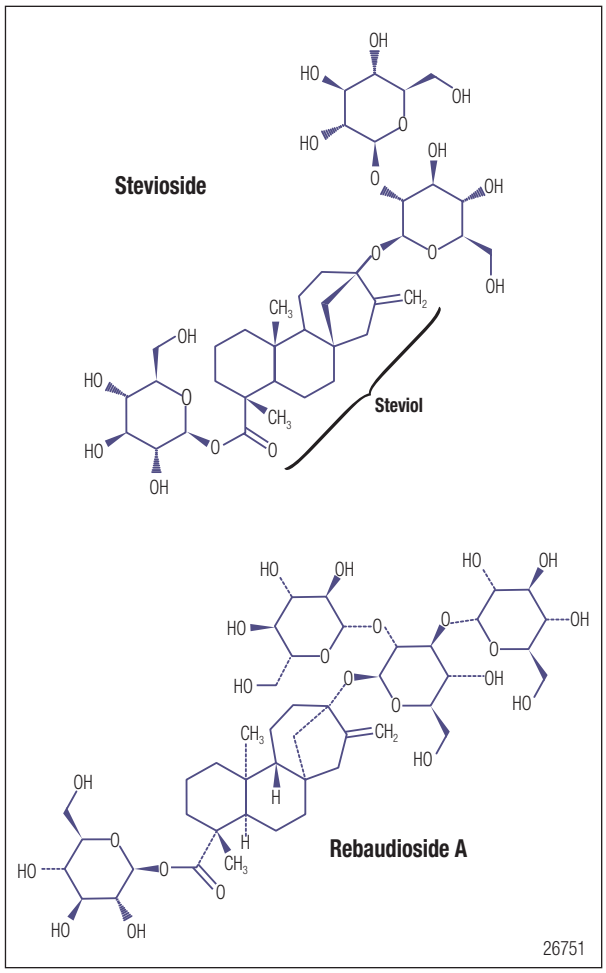

Figure 1. Chemical structures of stevioside and rebaudioside A.

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Typically, individual steviol glycosides are determined by liquid chromatography methods that require a polar amine column. The Joint European (FAO/WHO) Commission on Food Additives (JECFA) 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 determines the weight percentage of seven glycosides: stevioside, rubusoside, dulcoside A, steviolbioside, rebaudioside C, rebaudioside B, and rebaudioside A. The percentages (w/w) of glycosides present in the sample are normalized to a 0.5 mg/mL stevioside standard. This method specifies a 3.9–4.6 mm i.d. column format 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 USP rebaudioside A monograph in the food codex is similar to the JECFA monograph. The rebaudioside A monograph uses an acetate buffer to adjust the pH rather than phosphoric acid and a propyl-amine silane phase bonded to a silica gel column.[6] Because this method is intended to determine rebaudioside A, it is the only glycoside quantified. The method uses UV detection and a flow rate of 1.5 mL/min with a run time of 40 min resulting in 60 mL of mobile phase used per sample.

Analytical methods to determine these glycosides are challenging because the compounds do not absorb strongly in the UV region. Other detection methods, such as evaporative light scattering (ELS) can be used to improve steviol glycoside quantification. Here, steviol glycosides are determined in consumer sweeteners by UV and ELS detections. The glycosides are separated on the Thermo Scientific Acclaim Mixed-Mode WAX-1 column using 80/20 acetonitrile/ammonium formate buffer at pH = 3.0. This method uses the HILIC mode of the mixed-mode column, thereby allowing separation of multiple steviol glycosides. By using a volatile mobile phase, ELS detection is feasible, which adds further flexibility to the method for detection of glycosides that do not have strong UV extinction coefficients. In addition, the 2.1 mm i.d. column format reduces solvent use to 12.5 mL/sample compared to the range of 25–60 mL/sample for the JECFA and proposed USP methods.

Equipment

- Thermo Scientific Dionex UltiMate 3000 Intelligent LC system:
  - Thermo Scientific Dionex SRD-3200 solvent rack (P/N 5035.9250)
  - Thermo Scientific Dionex HPG-3200M pump (P/N 5035.0018)
  - Thermo Scientific Dionex WPS-3000TSL micro autosampler (P/N 5822.0025)
  - Thermo Scientific Dionex sample loop, 25 µL (P/N 6820.2415)
  - Thermo Scientific Dionex TCC-3200 column compartment (P/N 5722.0025)
  - Thermo Scientific Dionex VWD-3400 detector (P/N 5074.0010)
  - Thermo Scientific Dionex semi-micro PEEK flow cell, 2.5 µL (P/N 6074.0300)
- Varian 380-LC ELS detector
- Thermo Scientific Chromeleon 6.8 Chromatography Data System
- Thermo Scientific Dionex glass injection vials with caps and septa, 1.5 mL (P/N 055427) or
- Thermo Scientific Dionex polypropylene injection vials with caps and septa, 300 µL (P/N 055428)
- Thermo Scientific Nalgene filter unit, 0.2 µm nylon membrane, 1 L capacity (P/N 164-0020)

Reagents and Standards

- Deionized water, Type I reagent grade, 18 MΩ-cm resistivity or better
- pH buffer, 4.00 (VWR®, Radnor, USA, P/N BDH4018-500ML)
- pH buffer, 2.00 (VWR P/N BDH5010-500ML)
- Stevia standards kit, (ChromaDex®, Irvine, USA, P/N KIT-00019566-0HK) containing:
  - Isosteviol
  - Rebaudioside A
  - Stevioside
- Rebaudioside B (ChromaDex P/N 00018227)
- Formic acid (Sigma-Aldrich®, St. Louis, USA, P/N 06440)
- Ammonium formate (Sigma-Aldrich P/N 51691)
- Acetonitrile (Honeywell™, Morris Township, USA, P/N 015-4)

Samples

- Brand A: sweetener containing stevia leaf extract and inulin
- Brand B: sweetener containing rebiana (rebaudioside A) and erythritol

Conditions

Column:	Acclaim™ Mixed-Mode WAX-1 (5 µm), 2.1 × 150 mm (P/N 067084) Acclaim Mixed-Mode WAX-1 (5 µm), 2.1 × 10 mm guard column (P/N 069686) with guard holder (P/N 069580)
Mobile Phase:	80/20 acetonitrile/10 mM ammonium formate, pH = 3.0
Flow Rate:	0.5 mL/min
Temperature:	40 °C
Inj. Volume:	5 µL
Detection:	Variable Wavelength UV-vis Detector, 210 nm Evaporative Light Scattering Detector, N_2 flow, 1.2 SLM Nebulizer temperature, 35 °C Evaporator temperature, 60 °C PMT Gain, 2
System Backpressure:	~1100 psi
Noise:	~0.09 mAU (UV) ~0.7 mV (ELS)
Run Time:	25 min

Preparations of Solutions and Reagents

Mobile Phase Preparation

Transfer 0.63 g ammonium formate to a 1 L bottle and add 1000 g DI water. Adjust the pH of the resulting 10 mM ammonium formate solution to 3.00 ± 0.05 by adding 1700 µL formic acid. Filter the buffer through a 0.2 µm nylon filter unit to remove any insoluble particles that may be present.

Using the ammonium formate buffer described above, prepare a solution of 80/20 (v/v) acetonitrile/ammonium formate by transferring 200 g of the ammonium formate solution to a 1 L glass volumetric flask and bringing to volume with acetonitrile. This will yield 634 g of acetonitrile. Invert the flask to mix well. Do not top off the flask after mixing. Mixing formate and acetonitrile causes an endothermic reaction and the solution will cool substantially, resulting in a visible reduction in volume. Allow the solution to return to ambient temperature before using as a mobile phase.

Autosampler Syringe Wash Solution

To prevent carryover from the autosampler, a DI water wash solution was used.

Standards and Sample Solutions

Standards

Standard solutions of stevioside, rebaudioside A, rebaudioside B, and isosteviol were prepared by adding 2.0 mg to 1.0 mL of mobile phase to prepare a 2.0 mg/mL standard. This stock standard was then used to prepare standards of 0.06 mg/mL to 0.5 mg/mL of stevioside and rebaudioside A by appropriate dilution in the mobile phase.

Samples

Samples were prepared for analysis by extracting 0.25 g of sweetener with 10 mL of mobile phase. Brand A contains inulin, which is minimally soluble in organic solvents. The samples were mixed using a vortex mixer for a minimum of 20 s at least 4 times to dissolve the glycosides. Brand B fully dissolved followed by separation of erythritol, which was saturated in the mobile phase.

Precautions

Care must be taken to prepare the mobile phase consistently. Changes in either the ionic strength or pH of the mobile phase can lead to shifts in analyte retention times. If a decrease in resolution is observed, prepare the ammonium formate buffer again, paying close attention to the amount of ammonium formate added and to the final pH. If the pH is below 2.95, discard the solution and prepare another batch of buffer.

Using a premixed mobile phase will substantially decrease noise in the UV channel. Increased noise will result from online mixing of acetonitrile and the formate buffer that do not absorb equally in the UV range.

Results and Discussion

Separation

Figure 2 shows the separation of isosteviol, stevioside, and rebaudioside A on the Acclaim Mixed-Mode WAX-1 column. In Figure 2A, stevioside and rebaudioside A are easily observed by UV detection. Isosteviol does not have a strong UV extinction coefficient and is not detected. As seen in Figure 2B, isosteviol is easily detected by ELS. However, the concentration of isosteviol in the standard leads to detector saturation in the ELS channel. To calibrate isosteviol by ELS, 1 µL injections were used, reducing the amount on the column by a factor of five. Figure 3 shows the ELS detector response from a 1 µL injection of the same standard shown in Figure 2, which permits the quantification of isosteviol. In UV and ELS detection, the stevioside and rebaudioside A peaks are well-resolved, with no interferences.

Quantification Assay Linearity

Table 1 shows the linear relationship of peak area to concentration for rebaudioside A and stevioside using UV detection. The correlation coefficients for rebaudioside A and stevioside are 0.9974 and 0.9977, respectively, by UV detection.

Calibration curves using ELS detection are inherently nonlinear and were fit using exponential curves. This nonlinearity is the result of physical interactions that contribute to ELS detection; light scattering, reflection, and refraction by the particles each depend on the radius of the particle compared to the wavelength used for detection. Small particles predominately scatter but as the particles increase in size, reflection and refraction begin to impact the particle detection. Due to this, the peak area and concentration are related by the following exponential expression:

$$\text{Peak Area} = amb$$

where m is the sample mass and a and b are coefficients related to droplet size, concentration, and detection parameters. By plotting the concentration against the peak area, a curved line is observed. To obtain a straight line, a logarithmic plot is required. To fit the calibration curves for ELS detection, use the exponential fitting option available within the Dionex Chromeleon software. This calibration option best fits the data obtained in lieu of a log-log fit. Correlation coefficients reported within Dionex Chromeleon are from linear fits of converted data. These values are reported in Table 1.

Table 1. Calibration of Steviol Glycosides by UV and ELS Detections

Analyte	Detector	Range (mg/mL)	Linearity[a]	Calibration Type
Rebaudioside A	UV	0.06–0.50	0.9974	Linear Offset
	ELS	0.06–0.50	0.9920	Exponential
Stevioside	UV	0.06–0.50	0.9977	Linear Offset
	ELS	0.06–0.50	0.9930	Exponential
Isosteviol	UV	ND*	—	—
	ELS (1 µL injection)	0.03–0.25	0.9720	Exponential

* ND = not determined.

[a] Linearity reported for exponential calibration refers to the exponential plots as described in the text.

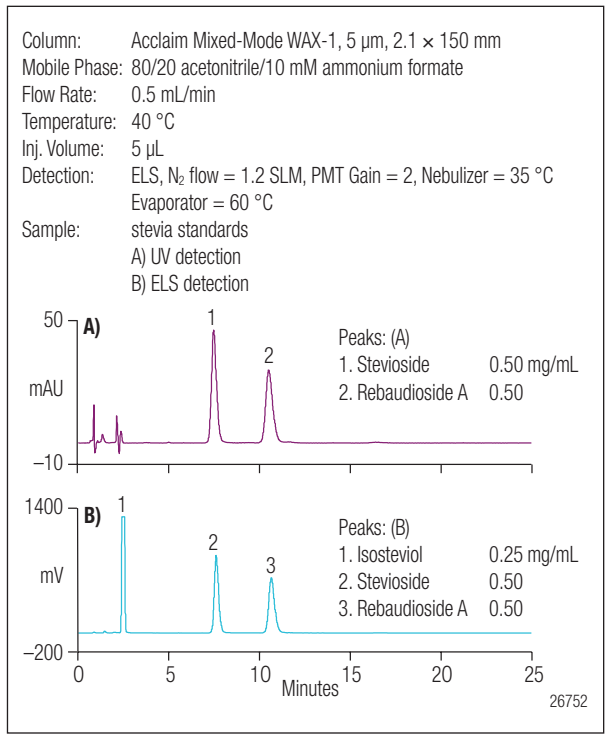

Figure 2. Separation of steviol glycoside standards on the Acclaim Mixed-Mode WAX-1 column.

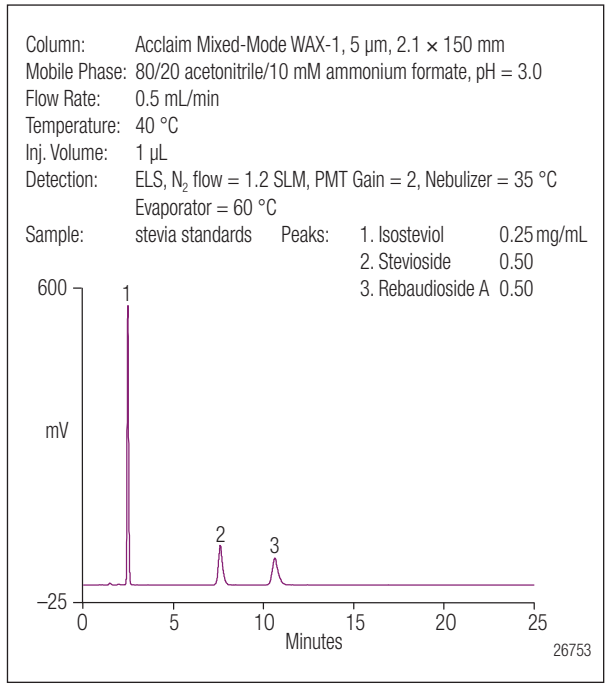

Figure 3. Isosteviol separated on the Acclaim Mixed-Mode WAX-1 column and detected by ELS.

Sample Analysis

Figure 4 shows the separation of an extract of a sweetener derived from extracted stevia leaves and dispersed in inulin. Several steviol glycosides are detected in this product. When analyzed by UV, which is a standard by the JECFA and USP monographs, four steviol glycosides are easily determined and baseline resolved: dulcoside A, stevioside, rebaudioside C, and rebaudioside A. When the sample is analyzed using ELS detection, there are a number of additional peaks not observed by UV (Figure 4B). The early eluting peaks, 1 and 2, are partially due to small amounts of inulin that dissolve in the mobile phase. This has been confirmed by an injection of an inulin sample. Stevioside and rebaudioside A are resolved from other peaks, and rebaudioside B is more easily detected than by UV detection. Both detection methods yield equivalent quantification of stevioside and rebaudioside A.

Figure 5 shows the separation of a 25 mg/mL solution of a commercial sweetener composed of rebiana. In this sweetener, the rebaudioside A is mixed with erythritol to dilute the glycoside and improve flavor. As seen in Figure 5A, when analyzing samples using UV detection, only rebaudioside A is observed. There is no evidence that this sweetener contains additional steviol glycosides. When using ELS detection (Figure 5B), both erythritol and rebaudioside A are observed, although the amount of erythritol present exceeds the dynamic range of the detector. The quantification of rebaudioside A is identical by both detection schemes.

Precision and Accuracy

Method precision was first tested by replicate injections of standards using two different batches of mobile phase. Table 2 summarizes the precision data. In both cases, the standards were properly quantified. Peak area RSDs range from 0.74 to 2.43, with the ELS peak area precision consistently higher than the UV peak area precision. This is a result of the nonlinear response in ELS that exaggerates small changes compared to UV. Retention time precision is excellent with retention time RSDs ranging from 0.05 to 0.14. Retention time variability between batches of mobile phase was within 1.1% for rebaudioside A. These data confirm that the method is reproducible with consistent mobile phase preparation.

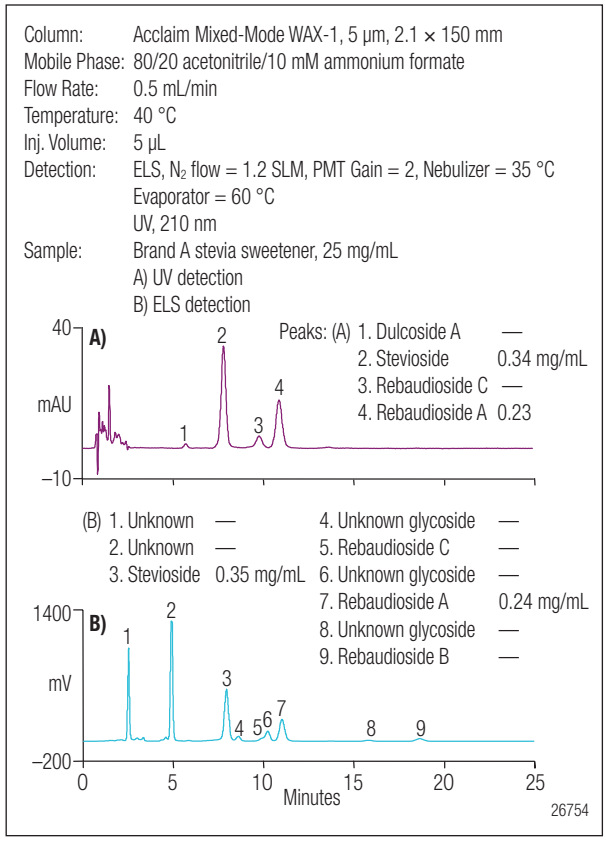

Figure 4. Separation of a stevia sweetener on the Acclaim Mixed-Mode WAX-1 column.

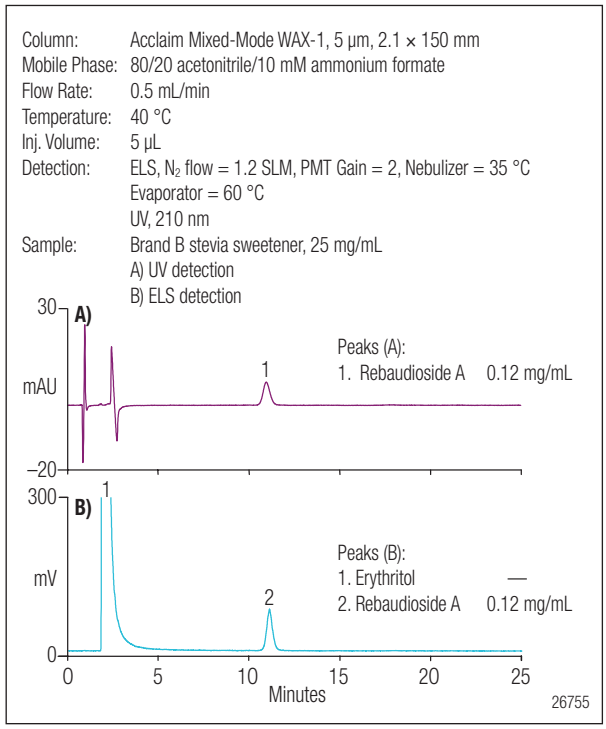

Figure 5. Determination of rebaudioside A in a commercial sweetener.

Samples of the two sweeteners were analyzed in triplicate over three days. Table 3 shows analyte quantification, retention time precision, and peak area precision data for Day 1 of sample analysis. The determined concentrations of glycosides in the samples are consistent by both UV and ELS detections. Table 4 shows the calculated mass of stevioside and rebaudioside A in a 1-g portion of the sweeteners along with intraday and between-day precision. During three days of analysis, Brand B was found to contain 4.0–4.3 mg/g of rebaudioside A. This corresponds to the expected amount of ~4 mg/g specified for use as a sweetener.[2]

Because it is an extract of stevia leaves without further purification to isolate an individual compound, Brand A contains higher concentrations of steviol glycosides compared to the rebiana sweetener. Table 3 shows determined concentrations in the dissolved sample. Similar to Brand B, the concentrations determined by UV and ELS detection methods are well-correlated. Table 4 shows the intraday precision for Brands A and B, which range from 0.5–6.3% from triplicate sample preparations.

Accuracy of the method was tested by spiking standards at known concentration into samples of Brand A and B sweeteners. The recoveries of stevioside and rebaudioside A ranged from 94.2–104%, suggesting that the method is accurate. The isosteviol recovery was lower at 90.6% due to the elution on the tail of erythritol in Brand A. However, isosteviol was not detected in any of the samples. Recovery of isosteviol in Brand B was evaluated, but close elution with a peak from the inulin interfered.

Table 2. Precision of Standard Injections (n=7, each standard at 0.20 mg/mL)

Analyte	Detection	Retention Time (min)	Retention Time Precision (RSD)	Peak Area Precision (RSD)	Amount (mg/mL)
Mobile Phase Batch 1					
Rebaudioside A	UV	11.08	0.05	0.79	0.18
	ELS	11.19	0.10	1.86	0.18
Stevioside	UV	7.81	0.03	0.74	0.18
	ELS	7.94	0.10	1.13	0.19
Isosteviol	UV	ND*	—	—	—
	ELS	2.61	0.08	1.47	0.22
Mobile Phase Batch 2					
Rebaudioside A	UV	10.96	0.09	1.54	0.18
	ELS	11.08	0.12	1.47	0.18
Stevioside	UV	7.75	0.12	1.09	0.18
	ELS	7.88	0.14	1.98	0.18
Isosteviol	UV	ND*	—	—	—
	ELS	2.60	0.07	2.43	0.23

* ND = not determined.

Table 3. Precision Values of Triplicate Analysis of Commercially Available Sweeteners, Day 1

Sample	Analyte	Detection	Retention Time (min)	Retention Time Precision (RSD)	Peak Area Precision (RSD)	Amount (mg/mL)
Brand A, Replicate 1	Rebaudioside A	UV	10.96	0.04	1.10	0.23
		ELS	11.08	0.07	2.00	0.25
	Stevioside	UV	7.74	0.05	0.36	0.32
		ELS	7.86	0.11	1.18	0.34
Brand A, Replicate 2	Rebaudioside A	UV	10.97	0.04	1.15	0.24
		ELS	11.10	0.07	1.19	0.25
	Stevioside	UV	7.74	0.05	0.91	0.32
		ELS	7.87	0.15	2.40	0.35
Brand A, Replicate 3	Rebaudioside A	UV	10.97	0.04	0.74	0.26
		ELS	11.11	0.04	0.04	0.28
Brand B, Replicate 1	Rebaudioside A	UV	10.95	0.06	0.94	0.10
		ELS	11.06	0.24	0.23	0.10
Brand B, Replicate 2	Rebaudioside A	UV	10.96	0.06	0.06	0.12
		ELS	11.09	0.07	1.17	0.12
Brand B, Replicate 3	Rebaudioside A	UV	10.96	0.04	0.81	0.11
		ELS	11.08	0.19	0.57	0.11

Table 4. Intra- and Interday Precision of Sweetener Analysis

Day/Sample	Analyte	Detection	Average mg Analyte/g Sweetener	Intraday Precision* (RSD)	Between-day Precision RSD
Day 1 Brand A	Rebaudioside A	UV	9.8	5.6	3.2
		ELS	10.4	6.3	3.5
	Stevioside	UV	13.3	6.2	4.0
		ELS	14.4	6.3	2.2
Day 2 Brand A	Rebaudioside A	UV	9.8	0.7	
		ELS	9.7	2.8	
	Stevioside	UV	13.5	0.5	
		ELS	13.8	1.0	
Day 3 Brand A	Rebaudioside A	UV	10.4	4.7	
		ELS	10.3	3.2	
	Stevioside	UV	14.3	4.4	
		ELS	14.1	1.5	
Day 1 Brand B	Rebaudioside A	UV	4.3	10.2	1.6
		ELS	4.3	9.0	3.4
Day 2 Brand B	Rebaudioside A	UV	4.2	1.5	
		ELS	4.1	2.0	
Day 3 Brand B	Rebaudioside A	UV	4.2	8.6	
		ELS	4.0	6.2	

* Precision values are normalized to the amount of steviol glycoside determined per 1 g of sweetener.

Table 5. Recovery of Standards Added to Stevia Sweetener Analysis

Sample	Analyte	Detection	Determined Amount with Spiking (mg/mL)	Amount Spiked (mg/mL)	Recovery (%)
Brand A	Rebaudioside A	UV	0.46	0.30	97.6
		ELS	0.48	0.30	104
	Stevioside	UV	0.52	0.30	95.3
		ELS	0.53	0.30	98.2
	Isosteviol	UV	ND	0.05	ND
		ELS	0.07	0.05	51.8*
Brand B	Rebaudioside A	UV	0.24	0.15	101
		ELS	0.25	0.15	103
	Stevioside	UV	0.14	0.15	94.9
		ELS	0.14	0.15	94.2
	Isosteviol	UV	ND	0.05	ND
		ELS	0.04	0.05	90.6

* Close elution of isosteviol with another component of the sweetener.

Conclusion

In this method, steviol glycosides are determined in consumer sweeteners by UV and ELS detections. The Acclaim Mixed-Mode WAX-1 column is used to resolve the steviol glycosides of interest. The method proposed requires only 12.5 mL of mobile phase sample compared to 25 to 60 mL/sample in the JECFA and proposed USP methods, respectively. This saves time and resources during mobile phase preparation, and reduces waste. Quantification of the two principal glycosides, stevioside and rebaudioside A, was precise and accurate by both detection methods. In addition, ELS detection has the added advantage of distinguishing additional components in the sample that are not UV absorbing, thus allowing for an additional method to check sweetener purity.

References

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5. Steviol Glycosides *Prepared at the 69th JECFA (2008), published in FAO JECFA Monographs 5, 2008.*
6. Rebaudioside A USPC, Inc. Dec 2008, http://forum.foodchemicaslcodex.org/online/pub/data/v200812/mon_pca_200812_f101747.xml

Suppliers

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