Comparison of Three Steviol Glycoside Methods that Utilize Different Chromatographic Columns and Mobile Phases

Kevin Welton*, Sharon Habeck, Eric Patterson and Wayne Ellefson
Covance Laboratories Inc., Madison, Wisconsin

Introduction

Use of the steviol glycoside rebaudioside A (Reb A) was granted a letter of no objection for use as a sweetener by the USFDA at the end of 2008. Reb A is intensely sweet (200-300 times sweeter than sugar) and has excellent stability and taste properties. It occurs naturally in the leaf of the stevia plant. With the high demand for carbohydrate reduction in certain foods while maintaining high standards of taste, Reb A and many other steviol glycosides or blends are expected to be used more widely. All of them will need testing to ensure they meet the minimum specifications for their use.

Presented here is a comparison of three HPLC methods for the determination of the purity of three Reb A lots. They are the Chinese National Standard – GB8270-1999, the JECFA method as published in FAO JECFA Monographs 10 (2010), and a Covance developed method. The methods each rely on different chromatographic columns and mobile phases to achieve resolution of Reb A from various impurities. The Covance and JECFA methods use reverse phase (C18) columns while the GB method uses an amine (NH_2) column. The Covance and the JECFA methods have the advantage of dissolving the material in a predominantly aqueous solution.

The results show that the Covance method identified the highest percentage (100.3%) of steviol glycosides with the other two methods identifying greater than 96%. All three methods identified eight known compounds (Reb A, Reb B, Reb C, Reb D, Reb F, Stevioside, Dulcoside A, and Steviolbioside). The JECFA method did not detect Rubusoside at levels greater than 0.1 wt/wt%, whereas the other two methods could. The Reb A results (wt/wt%) for the three lots are 54.7, 97.8, and 79.0 (Covance), 53.7, 98.1, and 75.4 (JECFA), and 54.4, 96.9, and 77.4 (GB). The results demonstrate that the different chromatographic conditions produce similar results for Reb A.

Methods and Experimental

Chinese National Standard – GB8270-1999

HPLC Column: NH_2, 4.6 x 150 mm, 5 µm
Mobile Phase: 80/20 Acetonitrile/water
Flow Rate: 1.2 mL/min.
Detection: UV 210 nm
Injection Volume: 15 µL
Calibration Type: Multi-point
LOQ: 25%*
Sample Preparation: 120 mg to 25 mL

*For the purposes of this comparison, the curve was extended through the origin to achieve a lower LOQ.

FAO JECFA Monographs 10 (2010)

HPLC Column: Capcell Pak C18 MG II, 4.6 x 250 mm, 5 µm
Mobile Phase: 32/68 Acetonitrile/10 mmole/L sodium phosphate buffer (pH 2.6)
Flow Rate: 1.0 mL/min.
Detection: UV 210 nm
Injection Volume: 5 µL
Calibration Type: Single-point
LOQ: 1% or 0.5%
Sample Preparation: 50 or 100 mg to 50 mL

Covance Method

HPLC Column: Synergi Hydro-RP, C18, 4.6 x 250 mm, 5 µm, two columns in series
Mobile Phase A: 0.10% Concentrated phosphoric acid
Mobile Phase B: Acetonitrile
Flow Rate: 1.5 mL/min.
Detection: UV 210 nm, reference wavelength 650 nm with 100 nm bandwidth
Injection Volume: 24 µL
Calibration Type: Single-point
LOQ: 0.1% for Reb A or other steviol glycosides or mixes
Sample Preparation: 125 mg to 50 mL
Gradient:

Time (min.)	%A	%B	Flow (mL/min.)	Comments
0.0	75.0	25.0	1.50	Initial
34.0	61.0	39.0	1.50	Linear
34.1	10.0	90.0	1.50	Linear
38.0	10.0	90.0	1.50	Hold
38.1	75.0	25.0	1.50	Linear
43.0	75.0	25.0	1.50	Hold

Accurate Measurement of Moisture

A sample of Reb A can rapidly gain weight when exposed to ambient humidity. A method may require the drying of a lot of Reb A prior to analysis; however, even in a time period as short as 15 minutes a sample of Reb A can gain over 2% in weight (Figure 1). Rapid absorption of moisture makes the accurate weighing of Reb A problematical. Under drier conditions Reb A will also quickly lose weight. Therefore all analyses presented herein were carried out on material that had been equilibrated at ambient conditions for one hour or more. Moisture content was accurately determined by Karl-Fischer titration using the equilibrated sample.

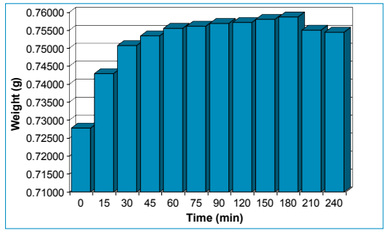

Figure 1. Rapid weight gain of Reb A when exposed to ambient humidity.

Results

All three methods produced similar results for the main component (Reb A) of each lot (Table 1). Each method produced data with a percent relative standard deviation (%RSD) of 0.4% or less for all three lots analyzed. When the data for each lot was combined for all three methods, a %RSD of 0.9% was calculated indicating consistency between methods.

Table 1.

Method	Reb A (%*) Lot A	Lot B	Lot C
FAO JECFA Monographs 10 (2010)	98.1	78.4	53.7
Chinese National Standard – GB8270-1999	96.9	77.4	54.4
Covance Method	97.8	79.0	54.7

*Reported on a dry-weight basis (wt/wt).

One additional consideration when determining the total amount of steviol glycosides in a lot is the method's ability to accurately quantitate analytes that are present in lesser amounts. A method that has a higher limit of quantitation (LOQ) may miss analytes present in smaller amounts. For the three methods under consideration the LOQ was determined as the percentage of the analyte that would produce a signal that is ten times the baseline noise following a typical sample preparation (Table 2, Figures 2-4).

Table 2.

Method	Calculated LOQ*	Injection Volume	Typical Sample Concentration
FAO JECFA Monographs 10 (2010)	0.908% or 0.454% if 100 mg of sample is analyzed	5 µL	1000 mg/L or 2000 mg/L
Chinese National Standard – GB8270-1999	25% (0.124% if the calibration curve is extended through the origin)	15 µL	4800 mg/L
Covance Method	0.115%	24 µL	2500 mg/L

*Calculated on a dry-weight basis (wt/wt).

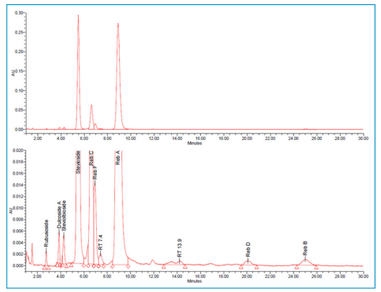

Figure 2. Chinese National Standard – GB8270-1999 method chromatogram of Lot C.

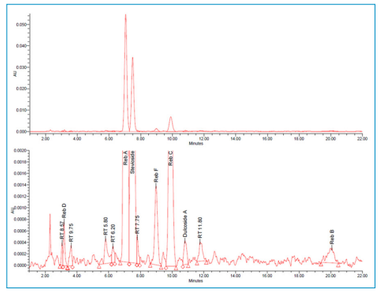

Figure 3. FAO JECFA Monographs 10 (2010) method chromatogram of Lot C.

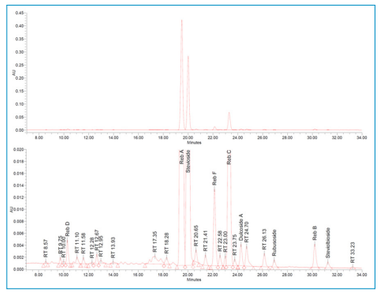

Figure 4. Covance method chromatogram of Lot C.

Methods having a higher LOQ may be accurate and reproducible yet miss minor constituents in a lot of Reb A and therefore underestimate the total sum of steviol glycosides. This is illustrated by lots B and C, which have lesser amounts of Reb A and correspondingly more of other steviol glycosides (Table 3). Yet the presence of minor constituents may contribute significantly to the products taste profile.

Table 3.

Method	Sum of Steviol Glycosides (%*) Lot A	Lot B	Lot C	%RSD Lot A	Lot B	Lot C
FAO JECFA Monographs 10 (2010)	99.3	94.7	94.9	0.2	0.3	0.4
Chinese National Standard – GB8270-1999**	98.7	96.3	96.9	0.3	0.3	0.3
Covance Method	100.3	99.5	99.8	0.3	0.3	0.4

*Reported on a dry-weight basis (wt/wt).
**Calibration curve modified to extend through the origin.

Considerations

One of the last eluting peaks for all three methods is Reb B. The three chromatograms in Figure 5 focus on the Reb B peak from an analysis of Lot C as performed by the three methods. The chromatograms presented are the same scale and were collected from the same HPLC system and UV absorbance detector. The Reb B peak is approximately 0.5% of the composition of Lot C. A clear advantage can be seen for using a gradient to maintain the sharpness of late-eluting peaks, and for designing an analysis to produce peaks with a favorable signal-to-noise ratio for important minor constituents near the LOQ of 0.5% or even 0.1%.

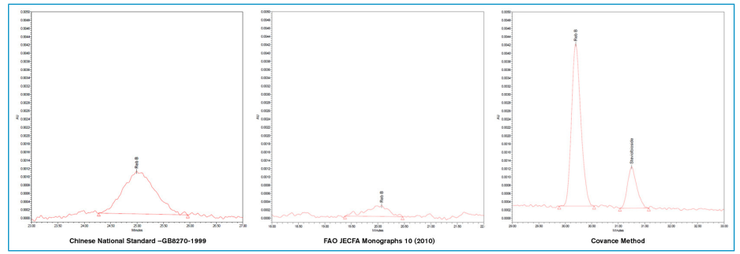

Figure 5. Comparison of the Reb B peak from three methods.

Both the JECFA and Covance methods make use of a single-point Reb A and a single-point stevioside calibration curve (Figure 6). The use of single-point calibration curves rather than multiple-point calibration curves is justified due to the highly linear response of the steviol glycosides over a very broad range of concentrations when using UV absorbance detection.

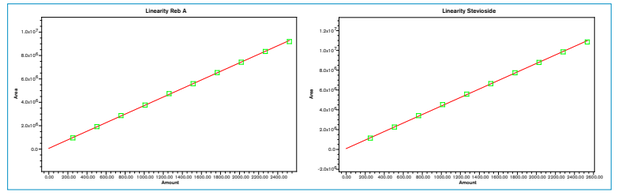

Figure 6. Linearity of steviol glycosides when using a single-point calibration curve.

Conclusions

While existing methods are suitable for measuring the main components of a lot of Reb A, they can have difficulty accurately quantifying steviol glycosides present in smaller amounts. Yet these components can account for several percent of the total. If they cannot be accurately quantified, the overall steviol glycoside content of a lot of Reb A can be underrepresented. These same minor constituents can also have a significant impact upon the taste profile of the lot. Therefore, a suitable method for the analysis of steviol glycosides should have a LOQ low enough to accurately quantify steviol glycosides other than the main component. The use of a gradient in the elution of steviol glycosides from the HPLC column can improve the response and peak shape of later-eluting analytes such as Reb B. It is critical to reduce the weight gained or lost due to moisture during sample preparation. The careful equilibration of lots of Reb A to ambient humidity followed by the accurate determination of moisture by Karl-Fischer titration may significantly improve the accuracy of sample analysis and standard preparation. A single-point calibration curve for Reb A and for stevioside is justifiable due to the highly linear response of the steviol glycosides analyzed using UV absorbance detection.

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Covance method MP-STEVIA.

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and Wayne Ellefson
Covance Laboratories Inc.
Madison, Wisconsin

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The Americas +1.888.COVANCE (+1.888.268.2623)
+1.609.452.4440
Europe/Africa +800.2682.2682 +44.1423.500888
Asia Pacific +800.6568.3000 +65.6.5686588

Web Site: www.covance.com

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