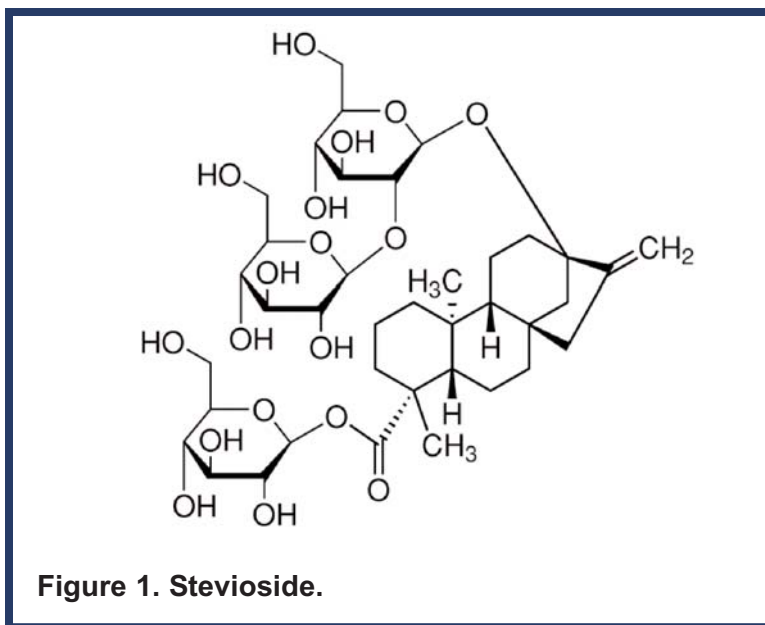


Analysis of Commercially Available Products Containing Stevia

The commercial use of the herb stevia refers to the extracts from the *Stevia rebaudina*, Bertoni plant. Although originally native to Paraguay and Brazil, it is now cultivated around the world. The plant contains at least eight diterpene glycosides ranging in both relative sweetness and abundance. For example, the two major glycosides stevioside (Figure 1) and rebaudioside A (Reb A) occur at ~ 3-10% and ~ 1-2% w/w, respectively in the dried plant. They are reported to be up to 250 times sweeter than sucrose.

In a few countries (e.g., Japan) extracts from the stevia plant have been used as a sweetener for centuries. However, up until recently, regulations in the United States prohibited the use of Stevia extracts in food or beverages, even though they could be purchased as nutritional supplements. This recently changed. In December 2008, the FDA issued GRAS affirmations for two commercial products containing Reb A: Truvia™ (Coca-Cola Company) and PureVia™ (PepsiCo). Both companies have also announced that beverages containing this sweetener will be available in 2009. The FDA GRAS affirmation does not provide approval for the use of all Stevia components in food products, just Reb A. Therefore, Stevia products must be highly purified and characterized prior to use.

This Application Note describes a routine gradient HPLC-Corona® Charged Aerosol Detection (CAD™) method that can be used to characterize Stevia components in raw and commercially available products. Universal detection has merits over the use of absorbance-based detectors for this analysis.



Method Parameters

Column:	Shiseido Capcell PAK C18 AQ, 4.6 x 250mm, 5µm
Temperature:	50 °C
Detector:	Corona CAD Plus
Nebulizer Heater:	On
Filter:	None
Mobile Phase A:	Deionized Water (DI), Acetonitrile, Trifluoroacetic Acid (TFA) (95:5:0.1)
Mobile Phase B:	Acetonitrile, DI (95:5)
Gradient Profile:	Table 1
Flow Rate:	1.0 mL/min
Injection Volume:	10 µL at 10°C
UV Wavelength:	210 nm

Time	% Mobile Phase B
0.0	5
3.0	5
30.0	90
35.0	90
40.0	5
45.0	5

Table 1. Gradient Profile.

Sample Preparation and Analysis

Commercially available products containing Stevia were purchased at a local supermarket. The products ranged in their classifications and recommended serving sizes (Table 2). The samples and standards were prepared in a aqueous methanol (20%) solution and sample injection concentrations are listed in Table 2.

The Stevia rebaudiana Bertoni leaves were purchased from Sigma-Aldrich (St. Louis, MO). The dry leaf extract was prepared by placing 83mg of dry leaf in 10mL of hot deionized water. The solution was then filtered through a 0.2µm Nylon filter and the filter was washed with an additional 15mL of the aq. methanol solvent. The solution was then diluted 3 fold in order to fall within the standard curve concentrations.

Standards of Reb A, Stevioside and Isosteviol were purchased from ChromaDex (Irvine, CA) and were certified to have > 97% purity. The stock solutions for Stevioside, Reb A, and Isosteviol were prepared at 167, 159 and 91ppm, respectively. The five point calibration curve solutions, ranging from 11 to 56ppm, as well as limit of detection (LOD) solutions were prepared by appropriate dilutions of the stock solutions. Two injections of each sample and three injections of the individual standards were analyzed. Injections of the standards and samples were interlaced over a three day period.

Product	Distributor	Classification	Serving Size	Injection
			1 packet (g)	Concentration (mg/ml)
Truvia [™]	Cargill, Incorporated	Table Sugar	3.5	5.9
PureVia [™]	Whole Earth Sweetener Company	Table Sugar	2	3.6
SweetLeaf Sweetener [™]	SweetLeaf	Dietary Supplement	1	2.6
Stevia Extract THE RAW [™]	Cumberland Packing Corp	Dietary Supplement	1	1.0
Stevia Supreme [™]	Stevia Company, Inc.	Dietary Supplement	1	1.1
SweetLeaf [®] Stevia Extract	Wisdom Natural Brands	Dietary Supplement	0.025	0.086

Table 2. Product Information.

Results and Discussion

Linearity, Reproducibility, Sensitivity and Accuracy

Both CAD and UV data were evaluated for linearity, sensitivity and accuracy. Three repetitive injections were made of the five different calibration solutions and the data obtained were analyzed for linearity and reproducibility. For the CAD, linear regression analysis was used (Figure 2); CAD and UV results are listed in Table 3.

Detector	Linear Correlation Coefficients	
	Rebaudioside A	Stevioside
CAD	0.9986	0.9978
UV @210	0.9992	0.9994

Table 3. Linearity Data.

The reproducibility of the analysis was evaluated by calculating the % RSD for repetitive injections of each point on the five point curves. The average RSD calculations for the CAD and UV detection are listed in Table 4.

Detector	Average % RSD	
	Rebaudioside A	Stevioside
CAD	0.77%	0.52%
UV @210	2.44%	1.75%

Table 4. RSD Data.

The sensitivity of the CAD and UV was evaluated by determining the LOD for the Stevioside and Reb A (Table 5). The CAD was far more sensitive than UV for all components of Stevia samples (see Figures 3 and 4).

Detector	Limit of Dection (mass on column)		
	Rebaudioside A	Stevioside	Isosteviol
CAD	4 ng	4 ng	80 ng
UV @210	65 ng	65 ng	> 900 ng

Table 5. Limit of Detection.

The accuracy of the analysis was evaluated two ways:

- By spiking a known amount of the standards into a sample and calculating recovery. The recovery for Reb A was 98.4% for the CAD compared to 92.2% recovery for the UV detector. Recovery of Stevioside was 103.4% for the CAD and 101.2% for the UV detector.

- From the manufacturer's reported value. The Reba reported value was 40% of Reb A in SweetLeaf Stevia Extract compared to a CAD value of 40.04% (representing 100.1% recovery).

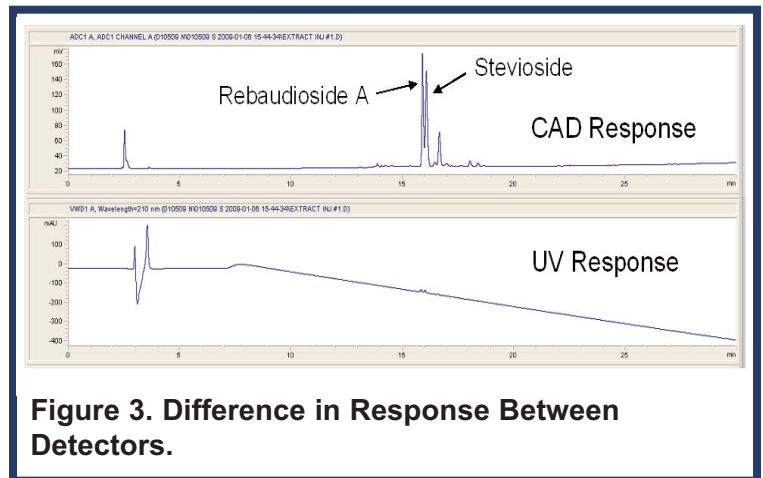


Figure 3. Difference in Response Between Detectors.

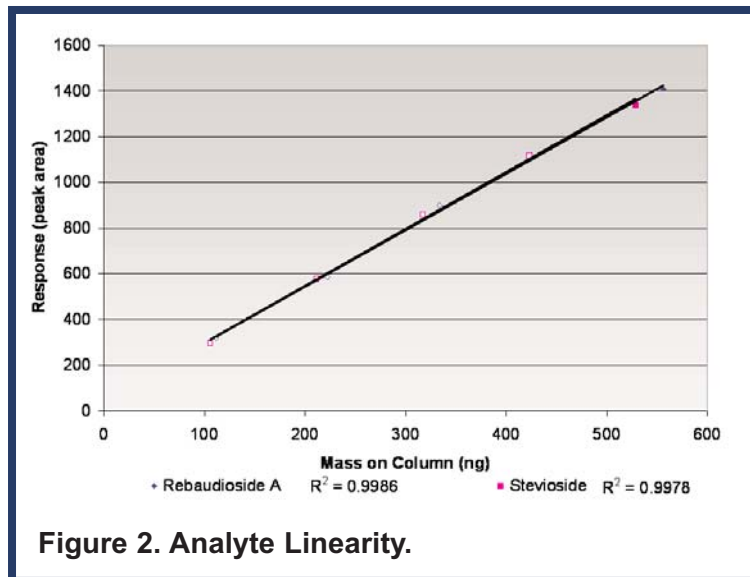


Figure 2. Analyte Linearity.

Rebaudioside A and Stevioside Concentrations in Commercial Products

Linear regression analysis of the five point calibration was used to calculate a w/w% of the two major glycosides in each of the products indicated in Table 2. All of the products contained Reb A and values were calculated and are illustrated in Figure 5 for both the UV and CAD. The Truvia[™], PureVia[™], and Stevia Extract "In the Raw"[™] are purified Reb A sweeteners and no Stevioside was detected.

The weight % of the Stevioside was calculated for each of the other products and is presented in Figure 6. The CAD shows good correlation to the

UV values obtained for all products (containing either all Reb A or similar concentrations of both Reb A and Stevioside) - between 99-105% CAD/UV. For the Stevia supreme and the Stevia leaves the Reb A correlation was 76% and 95%, respectively, while those of Stevioside were 108% and 94%, respectively. Unfortunately, the actual content of each component in these products is not available from the manufacturer, so comparison to calculated results is not possible.

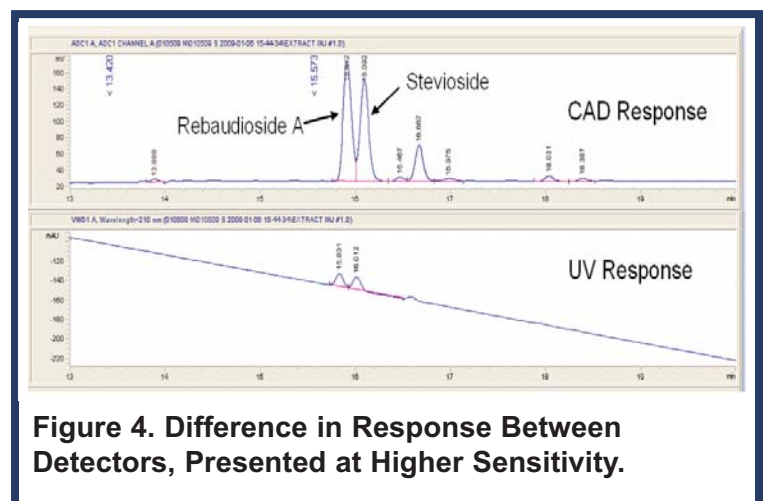


Figure 4. Difference in Response Between Detectors, Presented at Higher Sensitivity.

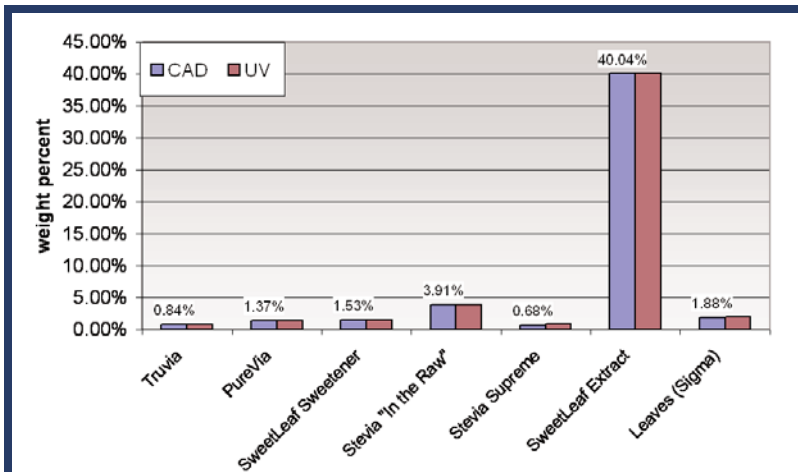


Figure 5. The Weight Percent of Rebaudioside A in 6 Commercially Available Stevia Sweeteners and the Dried Stevia Leaves Calculated using Corona CAD Detection and UV Detection @ 210nm. % Data for CAD.

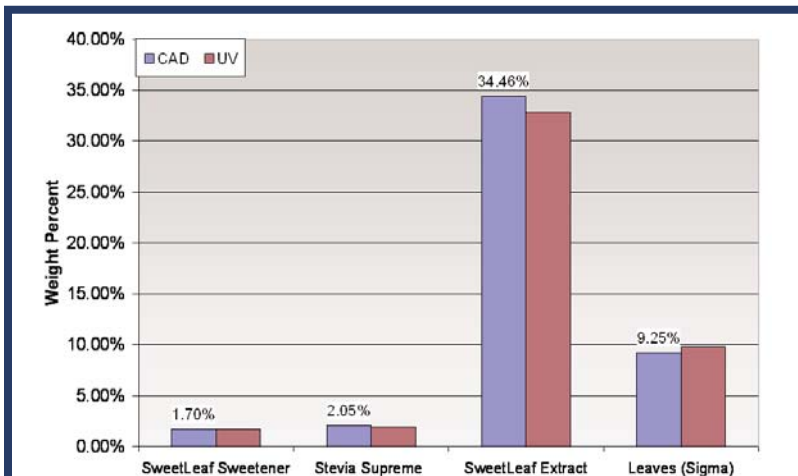


Figure 6. The Weight Percent of Stevioside in 3 Commercially Available Stevia Sweeteners and the Dried Stevia Leaves Calculated using Corona CAD Detection and UV Detection @ 210nm. % Data for CAD.

Stevia in Beverages

The FDA GRAS declaration now permits the commercial production of Stevia-based zero-calorie beverages. The Zieva company (Seattle, WA) began producing a line of alternative soft drinks with Stevia early in 2008 marketed as carbonated Stevia supplements. The Zieva Natural Cola and the Zieva Natural Twist flavors were purchased and diluted by diluting 1mL of each drink with 5mL of aq. Methanol solvent. The content of Reb A was calculated to be 0.016% in both drinks; the content of Stevioside was calculated to be 0.015% w/w in the twist and 0.016% w/w in the Zieva Natural Cola.

Isosteviol and Other Components

Isosteviol is a derivative of Stevioside and has been evaluated to determine whether there are any negative health implications when using Stevia sweeteners. This material possesses a weak chromophore and no signal was observed for the 90ppm standard by UV detection at 210 nm. On the other hand, the signal on the CAD was strong—the limit of detection was determined to be ~60ng on column. The compound was not detected in any of the commercial products tested.

As shown in Figures 5 and 6 the diterpene glycosides from Stevia constitute a small percentage of the table sugars' total mass. Other major components in Stevia products include sugar alcohols (Erythritol), dextrose, and Inulin soluble fiber. None of these compounds have strong UV chromophores but all can be detected by charged aerosol detection. In this method, these compounds are not retained—other CAD methods exist for their measurement.

The Corona[®] Charged Aerosol Detector



Conclusion

A gradient HPLC-UV-CAD method was developed for the routine analysis of sweeteners obtained from the Stevia rebaudina, Bertoni plant. The method is accurate, sensitive and reproducible.

Since the sweetness and potential aftertaste of the different diterpene glycosides is very important, many companies must ensure that their products are produced consistently using a viable analytical method. The Corona Charged Aerosol Detector has great applicability throughout the manufacturing process to ensure that product quality and consistency remains uniform. The Charged Aerosol detector's universality and enhanced sensitivity over UV detection allows for additional components from Stevia to be detected in foods and beverages.

Ordering Information

Corona Plus Charged Aerosol Detector	70-7041
Thermal Organizer Module	70-5499TA
Nitrogen generator	70-6003
Column, Capcell Pak Aq	88-92044



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