Isocratic Stevia Sweetener Analysis using Selective ZORBAX Columns

Application Note

Food

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Abstract

Two isocratic reversed-phase methods that have distinct selectivity, and use popular UV/MS or UV detection, are presented for analyzing Rebaudioside A and other steviol glycosides. A Rapid Resolution High Throughput (RRHT) Eclipse Plus Phenyl-Hexyl method using UV and MS detection resolved many compounds from a *Stevia rebaudiana Bertoni* plant extract instead of the other approach employing an amino column with a refractive index (RI) or other special detector. The RRHT Eclipse Plus Phenyl-Hexyl method's advantages include high peak capacity, isocratic mobile phase, low acetonitrile consumption, and unique selectivity compared to a ZORBAX Carbohydrate Analysis column method. Three commercially available Stevia sweeteners were analyzed for Rebaudioside A and Stevioside, the two major diterpenoid glycosides present in stevia leaves.

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Introduction

The use of stevia leaf derived sweeteners in food and beverages in the USA will likely increase, because the FDA has no objection to Rebaudioside A having GRAS (generally recognized as safe) status as a general purpose sweetener for food and drink. Stevia sweeteners may contain other steviol glycosides as well, mainly Stevioside, Rebaudioside C and Dulcoside A. The ratio of these stevia components influences the quality, identity and purity of the stevia extracts. Because the FDA GRAS confirmation is only for the use of Rebaudioside A at 95% purity or above in food and beverage products, stevia extracts must be highly purified and characterized prior to use [1-4].

Due to their lack of chromophores (Figure 1) many methods for sugars and similar compounds like steviol glycosides use refractive index, evaporative light scattering, or electrochemically pulsed amperometric detectors. Carbohydrate separation methods often use an amino bonded-silica based column too.

The method presented here takes advantage of the more prevalent UV diode array detector (DAD), and is coupled with an Agilent ZORBAX Eclipse Plus Phenyl-Hexyl Column instead of the more usual amino or carbohydrate specific column. The Eclipse Plus Phenyl-Hexyl method has a high aqueous, low acetonitrile, low UV, MS friendly mobile phase, so hydrolytic deterioration of the amino-silica bonded columns in a high aqueous environment is not a concern [5]. The end result is an uncomplicated, rugged, isocratic LC-UV-MS method for simple and complex stevia extract matrices.

An alternative method using an Agilent ZORBAX Carbohydrate Analysis column was also developed. It features significantly different selectivity compared to the ZORBAX Eclipse Plus Phenyl-Hexyl. This method is recommended as a secondary or confirmatory method for the ZORBAX Eclipse Plus Phenyl-Hexyl column, due to eventual hydrolytic deterioration of the amino silica bond, and also higher acetonitrile consumption. Nonetheless, it is still very useful for its different selectivity.

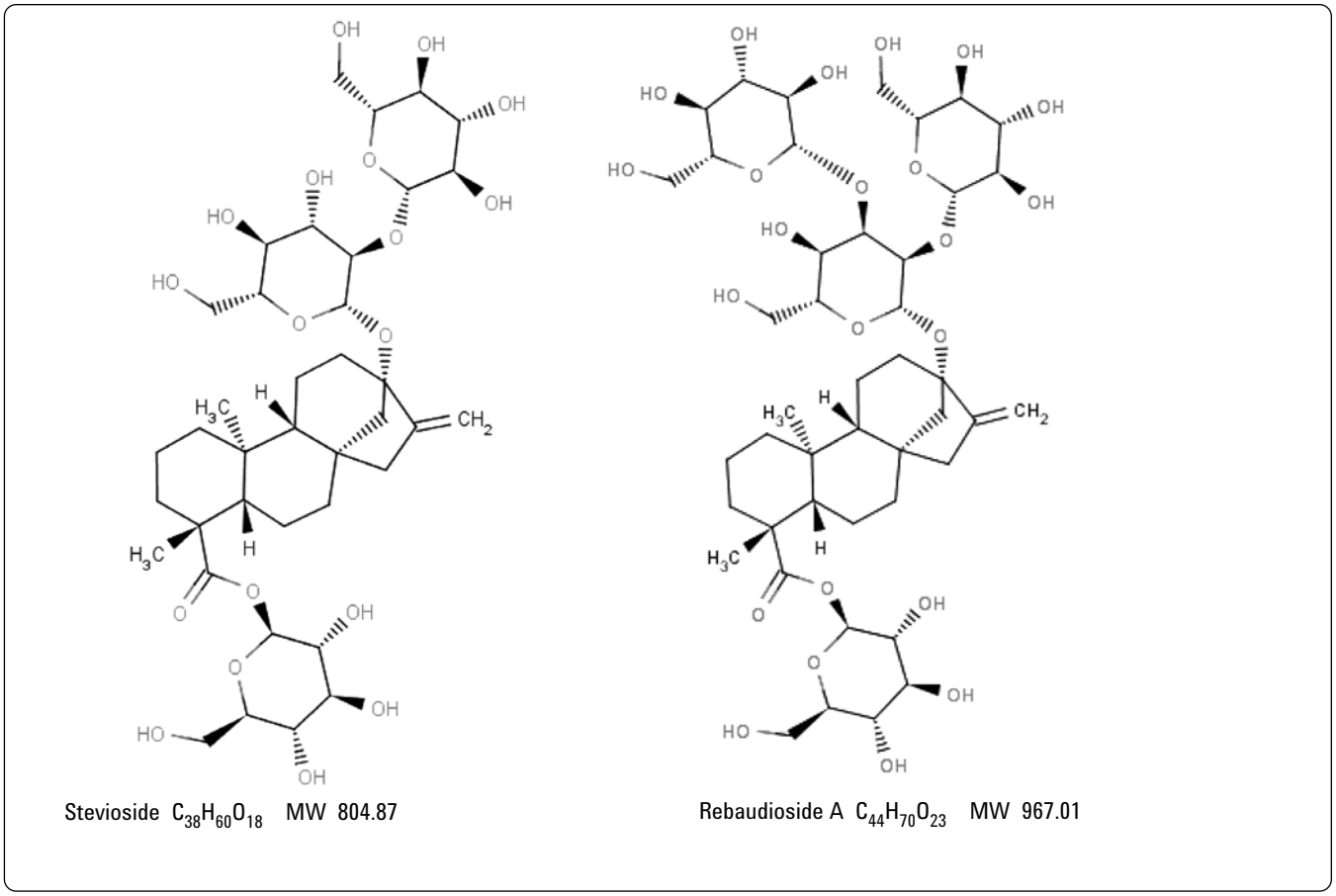

Figure 1. The two major diterpenoid glycosides present in Stevia rebaudiana Bertoni leaves.

Samples: Three retail sweeteners, all labeled as containing "stevia extract" were obtained from a local supermarket and diluted as follows:

1. A single-use powder packet (0.1 g/mL in H_2O)
2. A liquid concentrate (1:10 dilution in H_2O)
3. A stevia leaf powder extract (0.1g/mL in H_2O)

Standards: Stock solutions of Stevioside and Rebaudioside A reagent-grade standards from ChromaDex (Irving, CA) were made in water at concentrations of 2704 µg/mL (Stevioside) and 2867 µg/mL (Rebaudioside A). Further dilutions of the standard stock solutions were made to demonstrate linearity of the method. All samples were syringe filtered (0.2 µm) into autosampler vials for analysis.

ZORBAX Eclipse Plus Phenyl-Hexyl method

LCMS:	Agilent 1200SL with G1312B binary pump, 1316C DAD (diode array detector) and G1956B single quad MSD with electrospray ionization
Column:	ZORBAX Eclipse Plus Phenyl-Hexyl 2.1 mm x 100 mm, 1.8 µm p/n 959764-912
Spray chamber:	Drying Gas: 12 L/min, nebulizer pressure: 1811 torr, drying gas temp: 350 °C, capillary voltage: 3000V
MS parameters:	TIC scan: 300–3000 *m/z*, fragmentor: 70V, peak width 0.1, threshold 150, step size 0.1, gain 1.0
Mobile phase:	[A: B] Water (0.1% formic acid): ACN (0.1% formic acid) (82:18)
Flow rate:	0.42 mL/min
Detection:	204 nm and ESI-MSD
Temperature:	35 °C

ZORBAX Carbohydrate Analysis column method

LC:	Agilent 1200SL with G1312B binary pump and 1316C DAD (diode array detector)
Column:	ZORBAX Carbohydrate Analysis, 4.6 mm × 150 mm, 5 µm p/n 843300-908
Mobile phase:	[A: B] Water: ACN (22:78)
Flow rate:	1.5 mL/min.
Detection:	204 nm
Temperature:	30 °C

Results and Discussion

Two retail food sweeteners, labeled as dietary supplements, were analyzed on an Eclipse Plus Phenyl-Hexyl column. One was a powder packet, similar to sugar packets used for tea and coffee. The other was a stevia extract concentrated in ethanolic water. The ratio of the two major diterpenoid glycosides in the sweetener sources were quite different, with Rebaudioside A being more prevalent in the powdered sweetener, and Stevioside being dominant in the liquid (Figure 2). Rebaudioside A is considered the sweeter and better tasting of the two, although other minor glycosides may play a role in the overall taste quality. Rebaudioside A and Stevioside were identified with commercial standards and calibration curves were constructed, demonstrating linearity from about 100 to 3000 ppm (Figure 3).

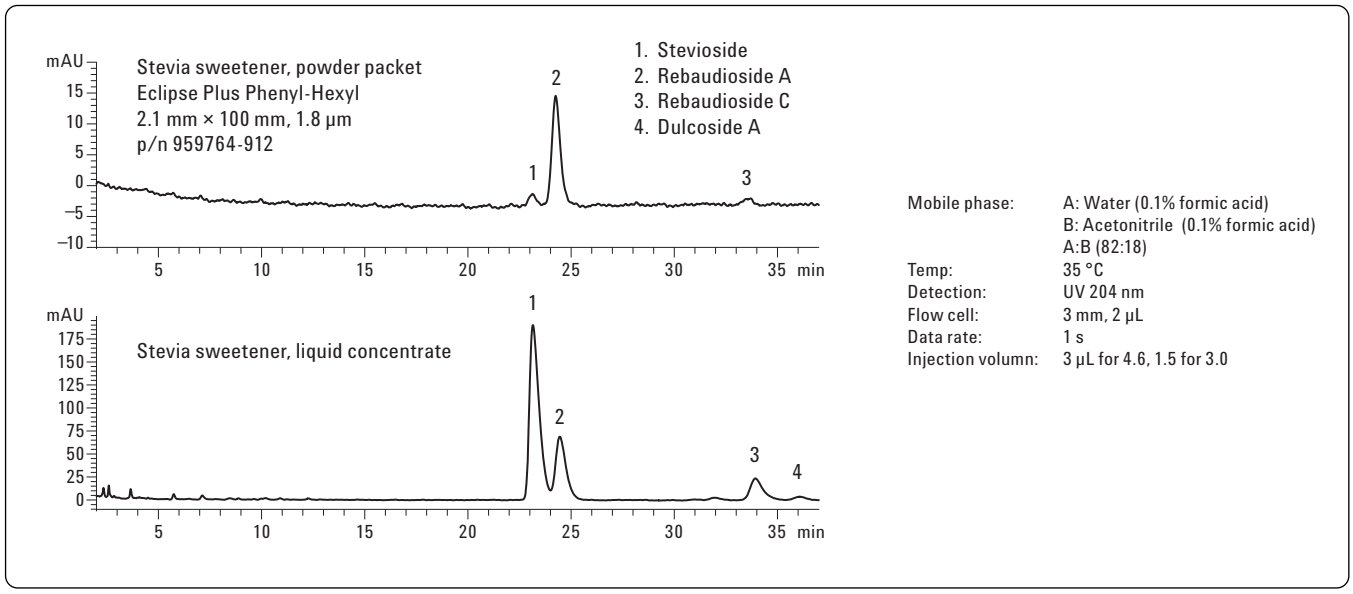

Figure 2. Steviol glycoside ratios differ between stevia sweeteners.

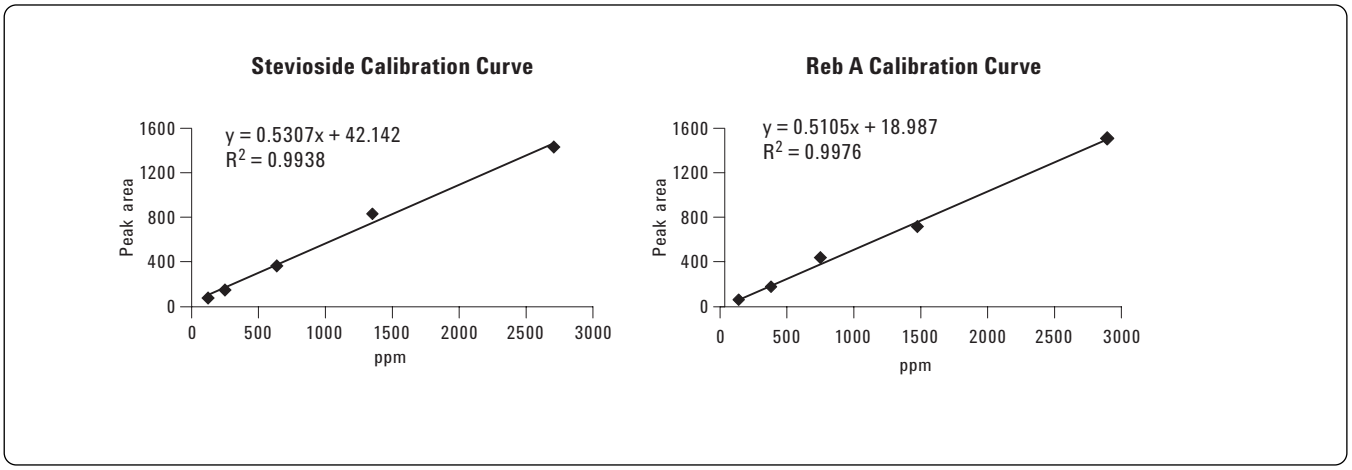

Figure 3. Linearity of Eclipse Plus Phenyl-Hexyl method for Stevia diterpenoid glycosides.

A third stevia extract, in powdered form, was analyzed and found to have many more components compared to the previous two samples. An Agilent 1200 Series LC/MSD SL G1956B Single Quad MSD was connected to the Agilent 1200 Thermostatted Column Compartment SL Plus G1316C DAD detector outlet to identify Dulcoside A and Rebaudioside C in addition to Rebaudioside A and Stevioside (Figure 4). In positive ionization mode, a pseudomolecular ion $[M+23]^+$ (sodium ion adduct) was used to identify peaks, in negative mode, the $[M-1]^-$ ion was used for identification. The negative mode resulted in higher ion abundance than the positive mode. The narrow bore column dimensions and formic acid (0.1% v/v) enhance electrospray ionization, and the isocratic mobile phase was well suited for ESI-MS, eliminating re-equilibration time and baseline drift common with gradients. It is also easy to transfer from one LC to another because delay volume associated with gradients is not a factor. The highly efficient RRHT Eclipse Plus Phenyl-Hexyl column contains 1.8-µm particles, and operated at a pressure of 400 bar under these conditions. This pressure was well within the operating limits of the RRHT column (600 bar) and the 1200SL LC (600 bar).

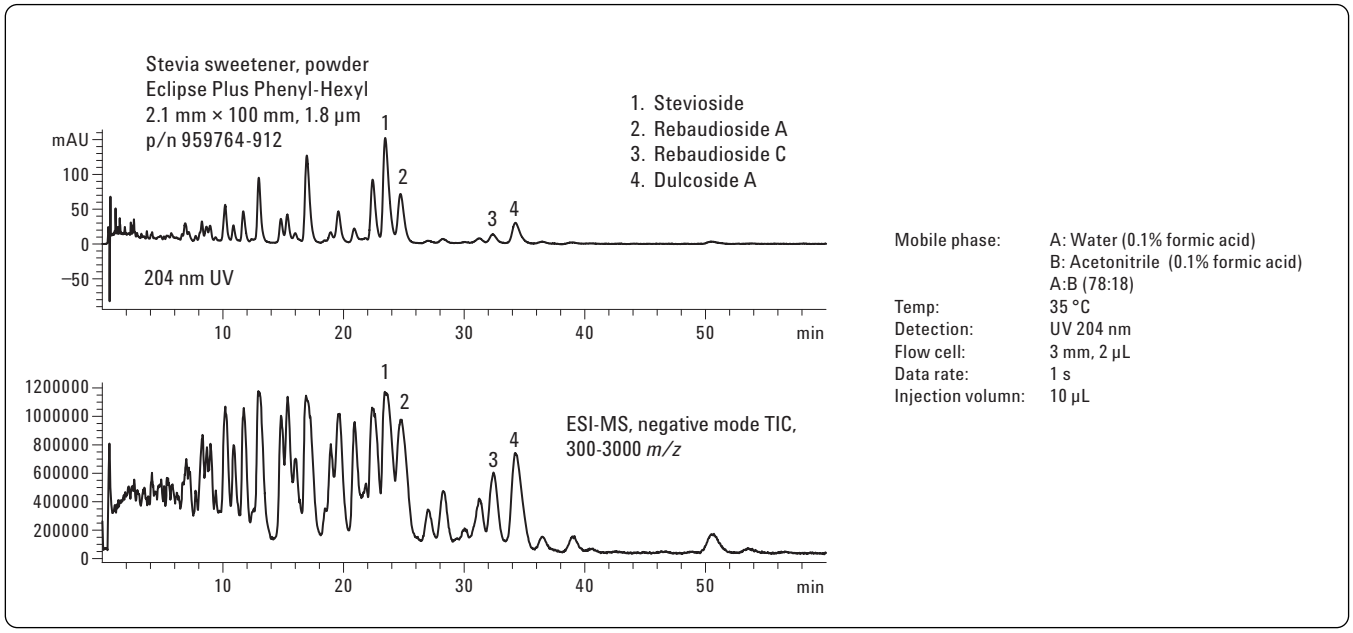

Figure 4. Isocratic analysis of complex Stevia leaf extract.

A supplementary steviol glycoside method was also developed using a ZORBAX Carbohydrate Analysis column with UV detection. The selectivity with this method was notably different compared to the RRHT Eclipse Plus Phenyl-Hexyl column method (Figure 5). The elution order was completely different. Other components of the stevia shown in Figure 3 however, were not as resolved on the Carbohydrate Analysis column, and some appeared to elute in the void volume, indicated by the large peak at t_0. This is likely due to differences in selectivity as well as efficiency between the two columns. The Carbohydrate Analysis column method was linear for Rebaudioside A and Stevioside over a 70–700 ppm range (Figure 6).

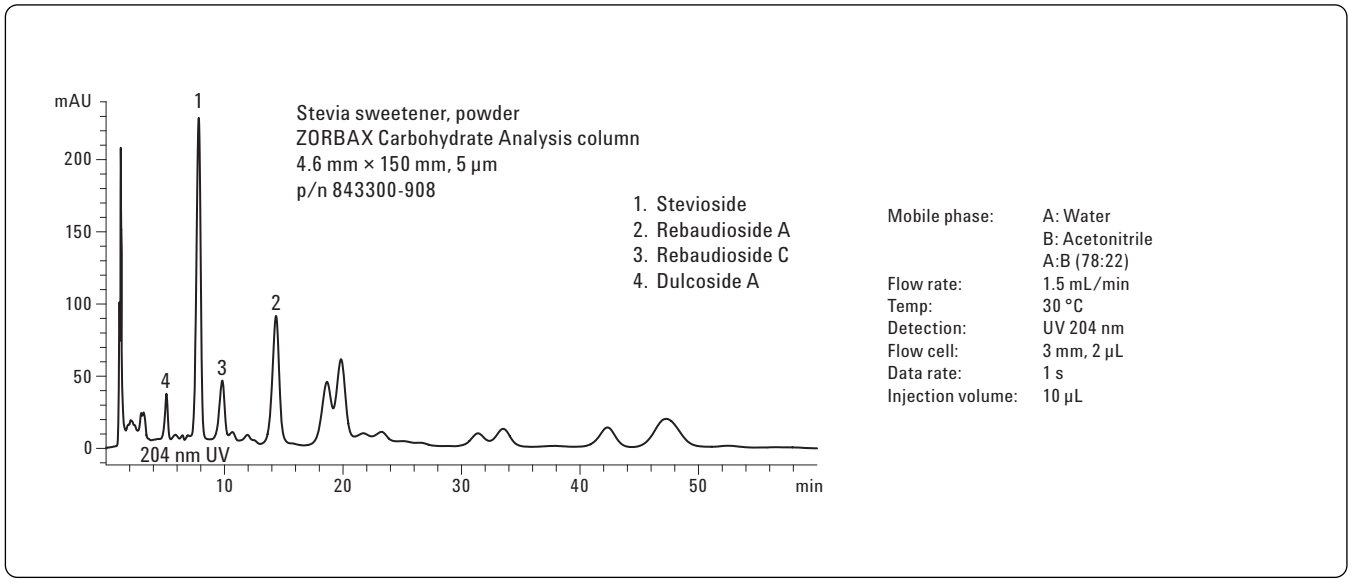

Figure 5. Elution order is completely different using the ZORBAX Carbohydrate Analysis column method.

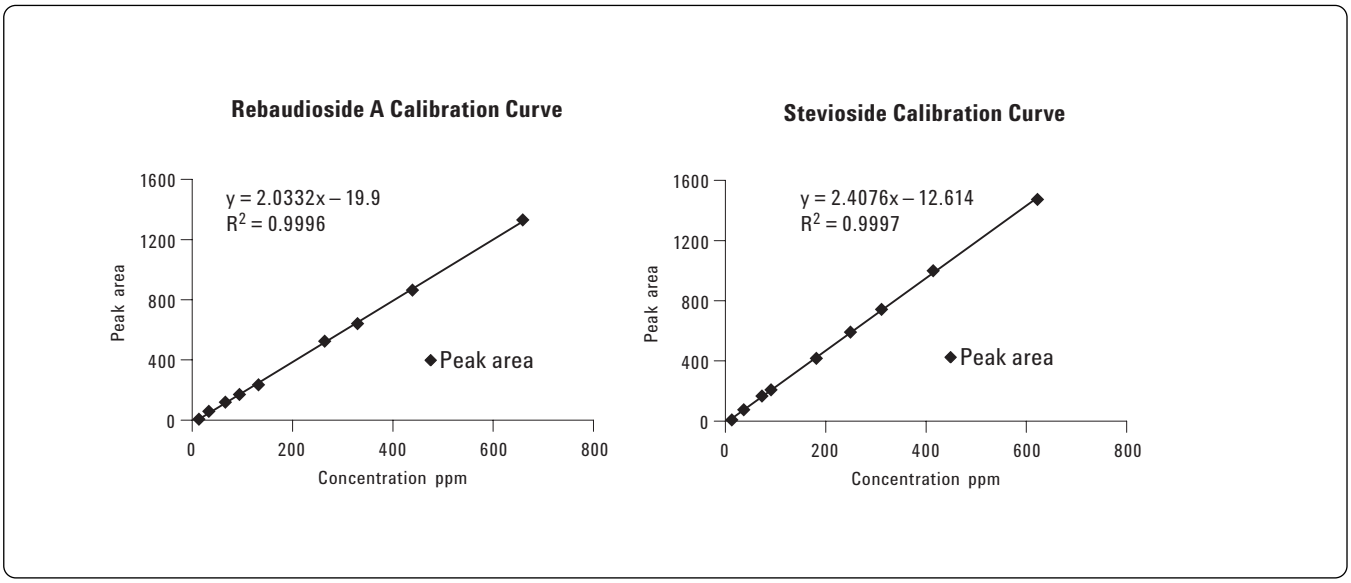

Figure 6. Linearity of ZORBAX Carbohydrate Analysis column method for Stevia diterpenoid glycosides.

Conclusion

The high efficiency and peak capacity of the RRHT Eclipse Plus Phenyl-Hexyl column combined with UV-MS detection suggests this method is useful for simple and complex stevia extracts. Benefits include using a very popular DAD and/or ESI-MS detector, compared to RI and other special detectors. Isocratic mobile phase advantages include no reequilibration time, and there is an ease of method transfer from LC system to LC system. The Carbohydrate Analysis column with UV detection method is a second possibility. The Carbohydrate Analysis column could be useful to confirm peak identity because the two columns have markedly different selectivity. The RRHT Eclipse Plus Phenyl-Hexyl method however, offers higher efficiency and more resolution.

References

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Printed in the USA
April 30, 2009
5990-3933EN

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