

An Improved HPLC Method for the Analysis of Diterpenoid Glycosides in *Stevia rebaudiana*



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ABSTRACT & INTRODUCTION

Stevia rebaudiana, also known as sweetleaf, has been used as a sweetener for centuries in South America. Extracts are up to 300 hundred times sweeter than table sugar, which is attributed to the diterpenoid glycosides, stevioside and rebaudioside A [1]. Although widely used in Japan and Brazil, most other countries do not allow the use of *Stevia* as a food additive. In the US, it can only be labelled as a dietary supplement. Many companies are now focusing on isolation of each of the individual diterpenoid glycosides, not just stevioside and rebaudioside A, for use in products as a low-calorie sugar alternative. Current HPLC methods for the determination of diterpenoid glycosides have been on NH₂-columns under isocratic conditions [2]. An improved HPLC method utilizing a Phenomenex Synergi Hydro-RP column and trifluoroacetic acid has been developed on a gradient reverse-phase system which overcomes the limitations of the reported NH₂ method with regards to separation of the minor components of *Stevia*. The method successfully separates rebaudiosides A (4), B (5), C (6), D (7) and F (8), dulcoside A (9), isosteviol (11), isosteviol monoside (12), steviol (1), steviol glucuronide (10), stevioside (3), steviolbioside (2), and several unidentified compounds.

Figure 1 [3]

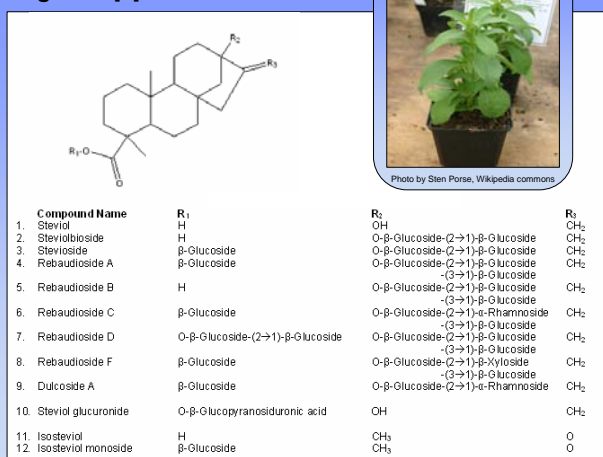


Photo by Sten Porse, Wikipedia commons

TABLE 1

Product Results Summary (mg/Serving)

	Serving Size	Reb. A	Stevioside	Reb. C	Reb. D
Sample A	0.25 mL	4.12	7.81	1.18	0.04
Sample B	NA*	6.87	15.56	2.25	0.18
Sample C	200 mg	81.48	24.95	9.25	5.39
Sample D	1 packet	2.28	2.71	0.69	1.03
Sample E	316 mg	3.87	12.23	1.44	0.07
Sample F	45 mg	1.76	1.98	0.49	0.68

Sample A is a commercially available liquid containing Stevia Extract dissolved in water, grain alcohol and glycerine.

Sample B is ChromaDex's Stevia Leaf (*Stevia rebaudiana*) BRM. *For comparison purposes, the serving size of the other leaf powder, 316 mg, was used in the above calculations.

Sample C is a commercially available Stevia Extract Powder, white in color, that claims to contain 90% Steviosides.

Sample D is a commercially available Sweetener Packet that claims to contain 85 mg of Stevia Leaf Extract per serving.

Sample E is a commercially available Stevia Leaf Powder, green in color, which has a serving size of 1/8 teaspoon.

Sample F is a commercially available Stevia Leaf Extract Powder, white in color, that claims to contain at least 80% Glucosylsteviosides.

EXPERIMENTAL

HPLC Agilent 1100 series equipped with a vacuum degasser, an autosampler injection system, a thermostated column oven, and a binary pump with a quaternary low pressure mixing valve.

Column Phenomenex Synergi Hydro-RP 250 x 4.6 mm, 5 μm particle size

Mobile Phase A 0.1% TFA in Milli-Q Water

Mobile Phase B Acetonitrile

Flow Rate 1.0 mL/minute

Temperature 60 °C

Injection Vol. 10 μL

Detection UV @ 202 nm

Gradient	Time, min.	%A	%B
	0	95	5
	3	95	5
	38	5	95
	40	5	95
	43	95	5

Re-equilibrate for 8 minutes.

Extraction

Stevia extracts were prepared at a concentration of 2 mg/mL. Plant material and table sweeteners containing excipient were prepared at a concentration of 20 mg/mL. Samples were sonicated in methanol for 5 minutes and centrifuged to pellet undissolved material, if necessary.

Figure 2 Mixed Rebaudiosides/Steviosides Standard

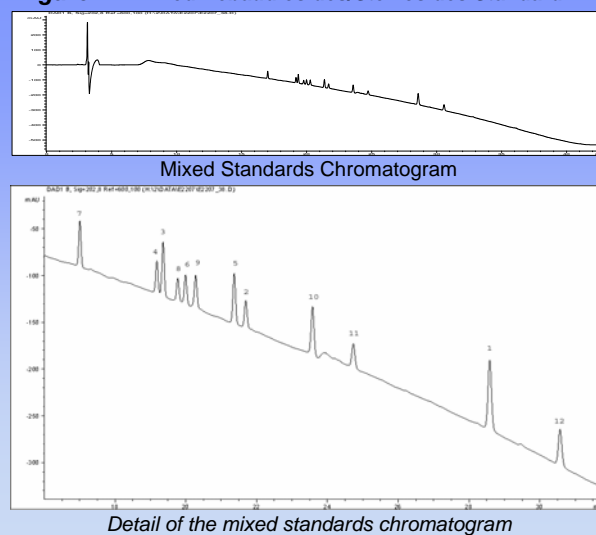
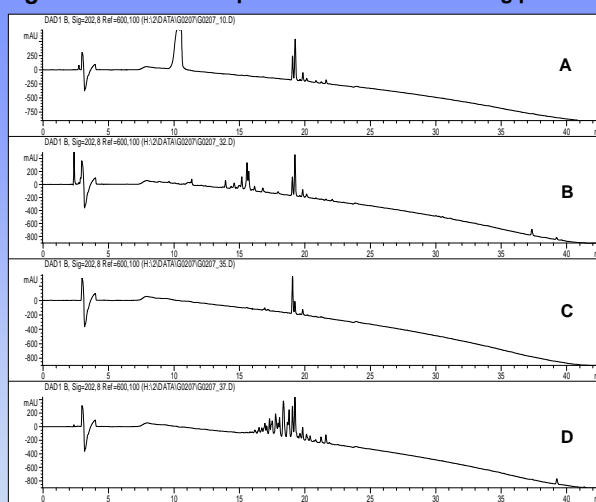


Figure 3 HPLC Comparison of Stevia-containing products



HPLC profiles of 4 samples: a glycerin suspension (A), ChromaDex Stevia Leaf BRM (B), Stevia Extract Powder (C), and a Stevia Extract Sweetener Packet (D).

DISCUSSION

• **Figure 3** illustrates differences between four of the samples tested. Of particular interest are the following:

- Sample C was the only extract or plant material tested that contained more rebaudioside A than stevioside.

- The multitude of peaks in Sample D may be due to the presence of maltodextrin in the formulation.

- There are a number of unidentified peaks in Sample B (eluting between 14 and 17 minutes) that may be evaluated as isolation targets.

- The improvements in reproducibility and equilibration time are advantages of this method.

• **Table 1** shows a summary of the results obtained for the samples tested, including those shown in Figure 4.

- The similarities of the HPLC profiles exhibited by Samples D and F, it is likely that a similar extraction process was used with differences resulting from the biomass.

- Sample C would likely be served well by the inclusion of a measuring device (scoop), to reduce the serving size to 10-20 mg. This reduced serving size would still likely be sweeter than 1 gram of sugar.

[1] Stevia. (2007, March 16). In *Wikipedia, The Free Encyclopedia*. Retrieved 17:45, March 16, 2007, from <http://en.wikipedia.org/w/index.php?title=Stevia&oldid=153675201>

[2] Kolb, N., Herrera, J.L., Ferreyra, D., Uliana, R.; *Analysis of Sweet Diterpene Glycosides from Stevia rebaudiana: Improved HPLC Method*. J. Agric. Food Chem. 2001, 49, 4538-4541.

[3] Wallin, H.; *Steviol Glycosides: Chemical and Technical Assessment* 63rd JECFA, 2004.