

Purification of Stevioside & Rebaudioside-A Using an Advanced Flash and Preparative Purification System

Introduction



Stevioside and Rebaudioside-A are major low-calorie diterpene steviol glycosides found in the leaves of *S. rebaudiana* and used as a sweetener in food and beverages. These compounds range in sweetness level from 250 to 450 times

sweeter than sugar. They are widely used as natural sweeteners for diabetic patients.¹

With advancements in instrumentation and purification media technologies, Automated Flash Column Chromatography (AFCC) and Preparative LC have become a highly versatile and efficient purification technique. Depending on the purification needs, AFCC can be used on its own to provide high quality purifications at low cost, or used as a complementary technique together with Preparative LC to enhance purity.

This application shows the benefit of new Reveleris® Prep purification system and Davisil® NNH2 purification media for the separation and isolation of the two main compounds, Stevioside and Rebaudioside-A, in *S. rebaudiana*. The flexibility of this new dual-mode Reveleris® Prep purification system allows the user to perform either flash or preparative LC or to combine both techniques to achieve the highest levels of purity and recovery.

Experimental

Extraction Conditions

Dried and ground leaves of *S. rebaudiana* (50 g) was placed in 1 L Erlenmeyer flasks and extracted by shaking for 30 min with 500 mL of EtOH 70% (w/w) at 70 °C. After cooling, extract was filtered and evaporated to dryness in vacuo (Yield 18% w/w).

HPLC Analysis

Stevioside and Rebaudioside-A standards were obtained from Sigma–Aldrich (St. Louis, MO) and analyzed by HPLC in order to determine the retention characteristics of the two compounds.

HPLC Chromatography Conditions

Column	Prevail™ NH ₂ Column, 4.6 x 250mm 5µ
Flow Rate	1.5 mL/min
Detector	ELSD and UV @ 210 nm
Run Time	11 minutes
Mobile Phase	A: Water (pH 5, adjusted with Acetic acid) B: ACN

ELSD Conditions

Temperature	60° C
Gain	1
Gas Flow	1.6

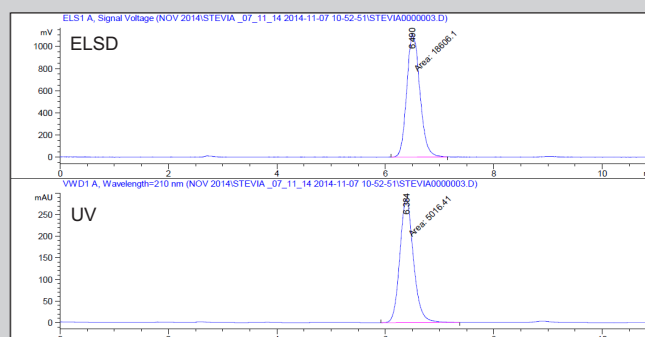


Figure 1. HPLC profiles of Stevioside standard.

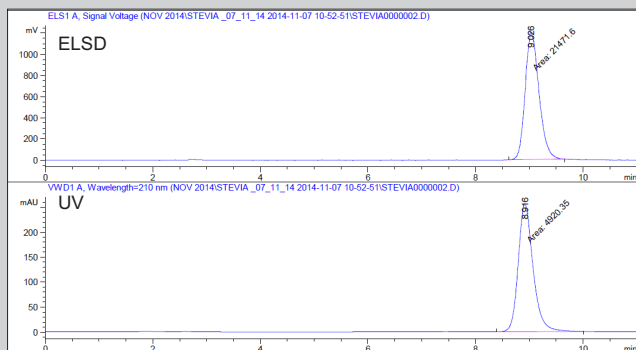


Figure 2. HPLC profile of Rebaudioside-A standard.

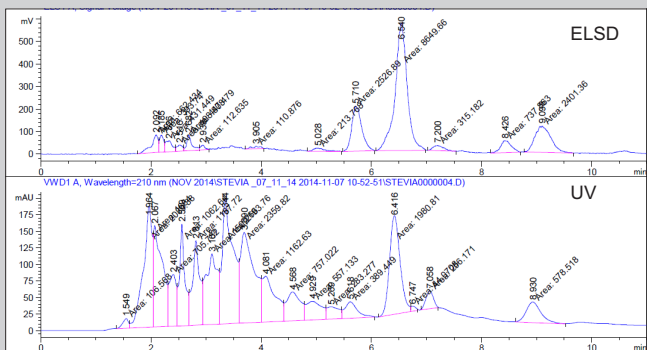


Figure 2. HPLC analysis of crude stevia extract with ELS and UV detection. ELS detection shows additional impurity peaks close to desired compounds which are not detected with UV.

Purification Using a Reveleris® Prep Purification System



The crude leaf extract was then purified using a two stage process to yield maximum purity of the two target molecules. Firstly the extract was purified by flash chromatography using an Amino bonded cartridge on the Reveleris® Prep purification system in flash mode. The sample was introduced using a 3g solid

load cartridge packed with the crude extract mixed with celite.

The fractions containing the target molecules were combined, and evaporated to dryness. Dried fraction was dissolved in 1.5 mL of water: acetonitrile (1:1) and injected onto a Prep LC column using the Reveleris® Prep purification system in prep mode.

For comparison a further sample was partly purified using the same flash chromatography method and then the combined fractions containing the compounds of interest were injected onto a conventional Agilent preparative LC system using the same Davisil® amino preparative LC column. The fractions containing the stevioside and the Rebaudioside-A were then collected and analyzed by HPLC to determine their purity and recovery.

Step 1: Flash Chromatography

Conditions

Sample	Stevia extract (500mg adsorb on celite)
Cartridge	Reveleris® Amino cartridge, 40g
Solvent A	ACN
Solvent B	Water
Flow Rate	30 mL/min

ELSD Detector

UV1 Wavelength	210 nm
UV2 Wavelength	254 nm
UV3 Wavelength	280 nm
Cartridge Equilibration	2 minutes
Injection Type	Dry Sample (3g loader)

Gradient

Step	Time (min)	%B
1	0.0	30
2	10.0	30

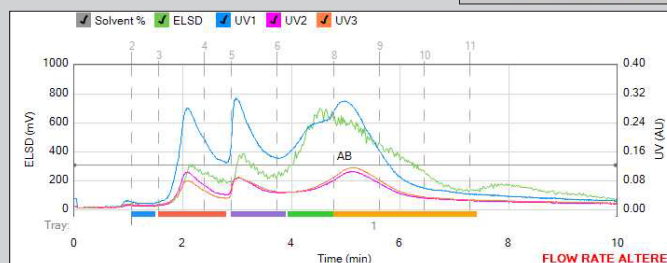
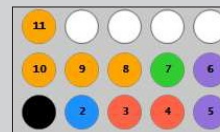


Figure 4. Flash chromatogram for stevia extract. Fractions 7-11 correspond to the mixture of Stevioside and Rebaudioside-A.

Step 2: Reveleris® Prep Purification

Conditions

Sample	Fractions 7-11 from step 1 (dissolved in ACN: water, 1:1)
Cartridge	Davisil® 710N NH ₂ Column, 250x25mm, 10-14µ
Solvent A	ACN
Solvent B	Water
Flow Rate	25 mL/min

ELSD Detector

UV1 Wavelength	250 nm
UV2 Wavelength	254 nm
UV3 Wavelength	280 nm
Column	5 min
Equilibration	
Injection Type	Liquid (10mL loop)

Gradient

Step	Time (min)	%B
1	0.0	20
2	43.0	20

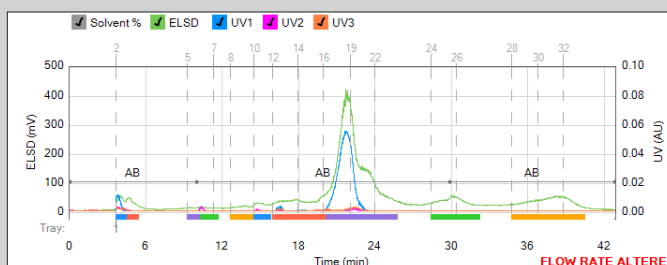


Figure 5. Prep chromatogram for combined fractions 7-11. Fractions 17-18 and 28-33 correspond to Stevioside and Rebaudioside-A.

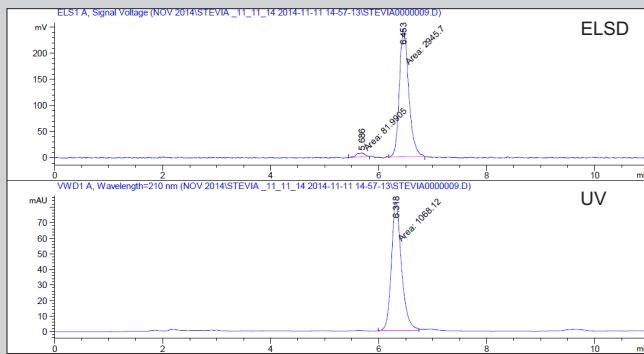


Figure 6. LC profile for combined prep fractions 17-18.

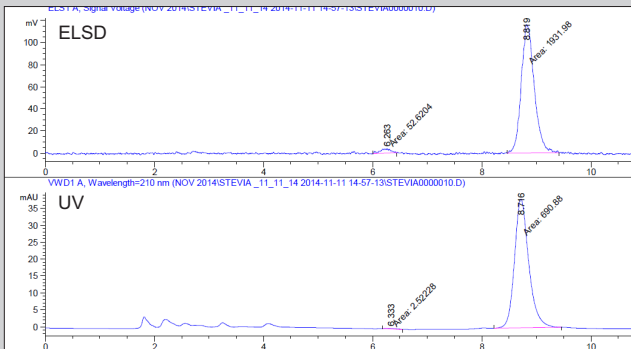


Figure 7. LC profile for combined prep fractions 28-33.

Step 2: Agilent Prep Purification

Sample	Fractions 6-11 from step 1 (dissolved in ACN: water, 1:1)
Cartridge	Davisil® 710N NH ₂ Column, 250x25mm, 10-14µ
Solvent A	ACN
Solvent B	Water
Flow Rate	25 mL/min
UV Wavelength	210 nm
Injection Type	Liquid (2mL loop)

Gradient

Step	Time (min)	%B
1	0.0	20
2	50.0	20

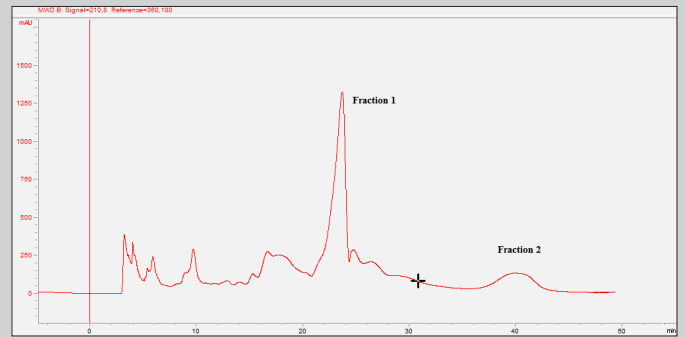


Figure 9. Preparative LC chromatogram for combined flash fractions 6-11. Fractions 1 and 2 correspond to Stevioside and Rebaudioside-A.

Purification Using an Agilent Prep System

Step 1: Flash Chromatography Conditions

Sample	Stevia extract (500mg adsorb on celite)
Cartridge	Reveleris® Amino cartridge, 40g
Solvent A	ACN
Solvent B	Water
Flow Rate	30 mL/min
ELSD Detector	
UV1 Wavelength	210 nm
UV2 Wavelength	254 nm
UV3 Wavelength	280 nm
Cartridge Equilibration	2 minutes
Injection Type	Dry Sample (3g loader)

Gradient

Step	Time (min)	%B
1	0.0	30
2	9.0	30

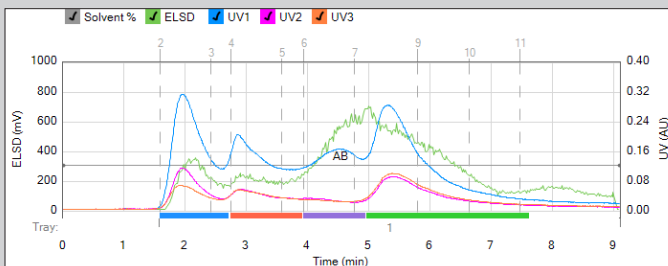
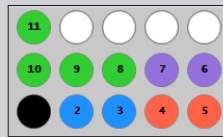


Figure 8. Flash chromatogram for stevia extract. Fractions 6-11 correspond to the mixture of Stevioside and Rebaudioside-A.

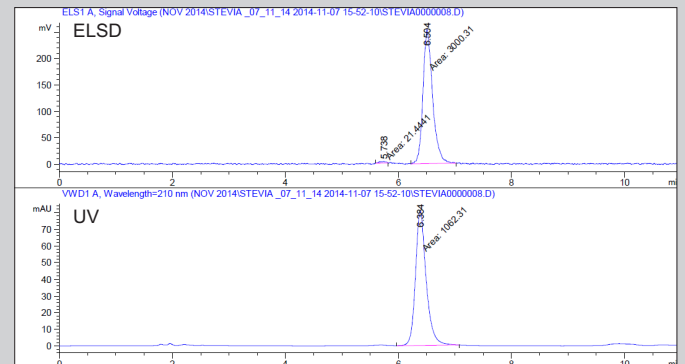


Figure 10. LC profile for prep fraction 1.

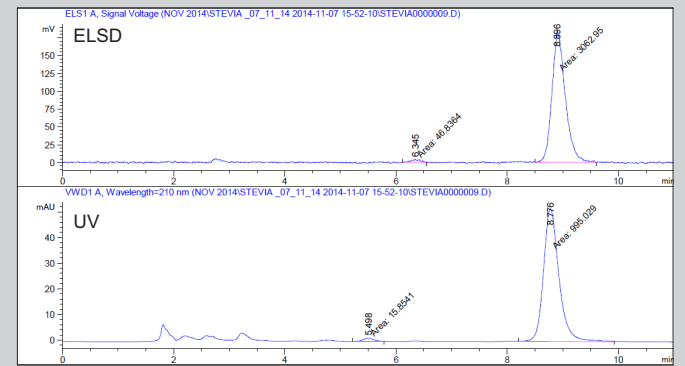


Figure 11. LC profile for prep fraction 2.

A summary of the purity and recovery of the two components is summarized in Table 1 below and compares the results obtained using the Reveleris® Prep purification system to undertake both the initial flash purification and the final prep LC purification with that obtained using a conventional Agilent preparative LC system for the final purification. The purities and recoveries were calculated using the HPLC chromatographic data on both the UV and ELSD signals.

Purity and Recovery Results



Purification System	Stevioside		Rebaudioside-A	
	Purity# (%)	Recovery# (mg/0.5g extract)	Purity# (%)	Recovery# (mg/0.5g extract)
Reveleris® Prep Purification System*	98.65	24.42	98.50	10.47
Agilent Prep System*	99.65	28.54	98.46	8.32
# Average of UV and ELSD Signals				
*Davisil® 710 NNH ₂ Media				

Table 1. Purity and recovery results of Stevioside and Rebaudioside-A.

Conclusion

It has been demonstrated that stevioside and Rebaudioside-A can be isolated and purified using the Reveleris® Prep purification system with UV and ELSD detection and Davisil® NNH₂ purification media.



For complex mixtures, the Reveleris® Prep purification system allows both flash and prep LC to be used for purification. This means that the relatively expensive prep LC column is protected from contamination and degradation by other components in the sample. These elements are retained or separated on the low cost flash cartridge.

In this application, the initial sample cleanup was done with flash using a Reveleris® amino cartridge, followed by a final preparative purification using Davisil® NNH₂ media. Comparison of the results also showed that the performance of the Reveleris® Prep purification system in preparative LC mode, performs as well as traditional preparative LC systems.

The flexibility of the Reveleris® Prep purification system allows the user to perform either flash or preparative LC or the ability to combine both techniques on the same system to achieve the highest levels of purity and recovery.

References

1. Genus, J.M.C.; Augustijns, P; Mols, R.; Buyse, J.G.; Driessen B.; Metabolism of Stevioside in pigs and intestinal absorption characteristics of Stevioside, Rebaudioside-A and steviol. Food and Chemical Toxicology, 41 (2003), pp. 1599-1607.

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