IMPROVING CHROMATOGRAPHIC RESOLUTION OF THE JECFA METHOD FOR THE ANALYSIS OF STEVIOL GLYCOSIDES

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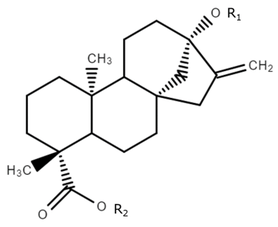
INTRODUCTION

Steviol glycosides (SG) are often used as non-caloric sweeteners in foods and beverages. There are more than 40 SG have been identified. The most abundant ones are rebaudioside A (Reb A) and stevioside (SV). However, some minor SG are becoming more readily available and are in demand due to their higher sweetness intensity and less bitter aftertaste.

The Food and Agriculture Organization of the United Nations, and the World Health Organization (FAO/WHO) Joint Expert Committee on Food Additives (JECFA) has recently published a SG monograph, in which a reversed phase LC was recommended for the determination of the major and the minor SG by LC-UV and LC-UV-MS, respectively. However, the chromatographic resolution is not adequate enough.



Compound		R_1	R_2
Rebaudioside E	Reb E	Glcβ(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-
Rebaudioside D	Reb D	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-
Rebaudioside N	Reb N	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside M	Reb M	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside I	Reb I	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-3)Glcβ1-
Rebaudioside A	Reb A	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ1-
Stevioside	SV	Glcβ(1-2)Glcβ1	Glcβ1-
Rebaudioside F	Reb F	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ1-
Rebaudioside C	Reb C	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ1-
Dulcoside A	Dul A	Rhaα(1-2)Glcβ1-	Glcβ1-
Rubusoside	Rub	Glcβ1-	Glcβ1-
Rebaudioside B	Reb B	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	H-
Steviobioside	SVB	Glcβ(1-2)Glcβ1-	H-
Rebaudioside O	Reb O	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-
IsoRebaudioside A*	IsoReb A	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ1-

*: Same as Reb A except that the double bond is located between C15 and C16.

Figure 1. Structures of steviol glycosides. R_1 and R_2 for specific steviol glycoside compounds are listed in the table.

OBJECTIVE

The goal of this work is to improve the chromatographic resolution of the RPLC of SG, without extending the run time.

EXPERIMENTAL

LC conditions

LC System: Arc™ Premier System (BSM) with a 2998 PDA Detector

Detection: UV (210 nm) and PDA (200 - 400 nm)

Software: Empower™ 3 CDS

Mobile phases: A: Acetonitrile/water (2:8 v/v, with 0.02% formic acid).
B: Acetonitrile (with 0.02% formic acid)

Col Temp.: 45 °C

Separation using 2.5 µm Particle Column:

Column: XSelect™ Premier HSS T3 VanGuard™ FIT Column, 130Å, 2.5 µm, 4.6 mm X 150 mm (p/n 186009863)

Inj. Vol.: 10.0 µL

Gradient program:

Time (min)	Flow rate (mL/min)	A (%)	B (%)	Curve
Initial	0.7	95.0	5.0	-
1.80	0.7	95.0	5.0	6
19.50	0.7	72.5	27.5	6
21.80	0.7	72.5	27.5	6
22.00	0.7	50.0	50.0	6
25.50	0.7	50.0	50.0	6
25.60	0.7	95.0	5.0	6
30.00	0.7	95.0	5.0	6

Separation using Sub-2 µm Particle Column:

Column: ACQUITY™ UPLC™ HSS T3 Column, 100Å, 1.8 µm, 3 mm X 150 mm (p/n 186004681)

Inj. Vol.: 5.0 µL

Gradient program:

Time (min)	Flow rate (mL/min)	A (%)	B (%)	Curve
Initial	0.4	95.0	5.0	-
1.35	0.4	95.0	5.0	6
14.50	0.4	72.5	27.5	6
16.20	0.4	72.5	27.5	6
16.40	0.4	50.0	50.0	6
19.00	0.4	50.0	50.0	6
19.10	0.4	95.0	5.0	6
24.00	0.4	95.0	5.0	6

METHOD DEVELOPMENT

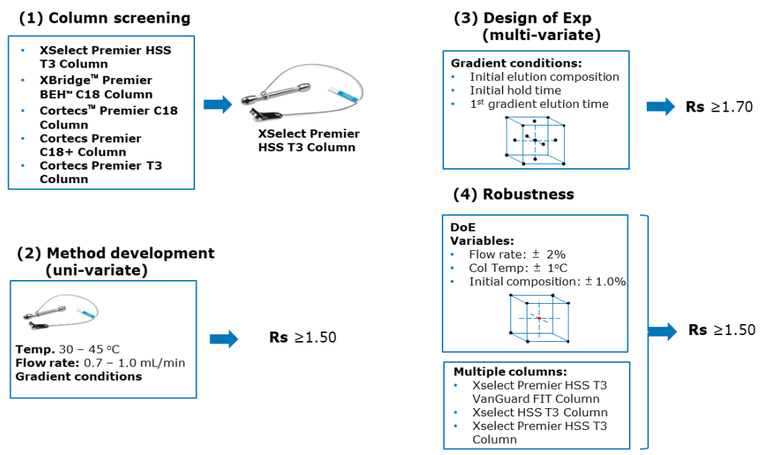


Figure 2. *Using the enhanced approach to method development as recommended by the ICH Q14 Analytical Procedure Development guideline.*

RESULTS

1) Chromatography optimization

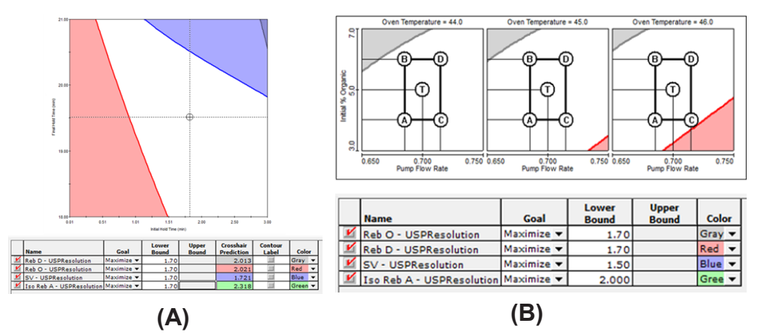


Figure 3. *(A) Acceptable performance region from Design of Experiment; (B) Acceptable performance regions from DoE robustness test.*

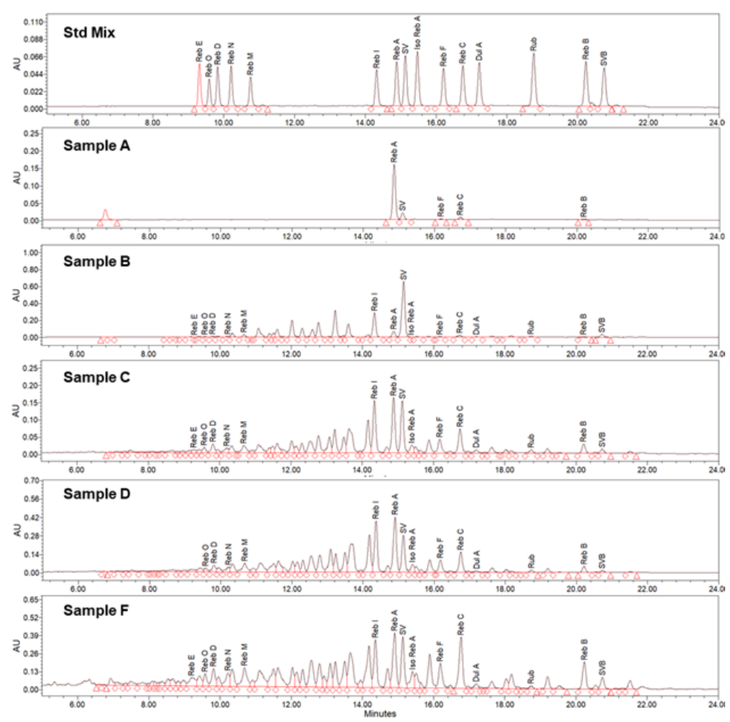


Figure 4. *HPLC-UV chromatograms of 15 steviol glycoside standard mixture and stevia extract products (XSelect Premier HSS T3 VanGuard FIT Column, 2.5 µm, 4.6 mm X 150 mm).*

2) Analytical performance

Linearity: ≥ 0.999

LOQ: 0.001—0.004 mg/mL

Recovery: 95.9% - 111.8%

Table 1.A

SG	Equation	R^2	LOQ (mg/mL)
Reb E	$Y = 2.60 \times 10^5 X + 6.22 \times 10^2$	0.9994	0.002
Reb O	$Y = 1.64 \times 10^5 X + 1.89 \times 10^2$	0.9993	0.001
Reb D	$Y = 2.47 \times 10^5 X + 3.12 \times 10^2$	0.9993	0.004
Reb N	$Y = 2.43 \times 10^5 X + 1.67 \times 10^3$	0.9995	0.003
Reb M	$Y = 1.88 \times 10^5 X + 3.48 \times 10^2$	0.9994	0.004
Reb I	$Y = 2.47 \times 10^5 X + 1.16 \times 10^3$	0.9992	0.003
Reb A	$Y = 3.02 \times 10^5 X + 3.82 \times 10^3$	0.9995	0.003
SV	$Y = 3.50 \times 10^5 X + 3.30 \times 10^3$	0.9992	0.002
Iso Reb A	$Y = 3.78 \times 10^5 X + 3.27 \times 10^3$	0.9994	0.004
Reb F	$Y = 2.70 \times 10^5 X + 7.15 \times 10^2$	0.9993	0.004
Reb C	$Y = 2.95 \times 10^5 X + 2.04 \times 10^3$	0.9994	0.001
Dul A	$Y = 3.22 \times 10^5 X + 1.71 \times 10^3$	0.9993	0.002
Rub	$Y = 4.26 \times 10^5 X + 4.33 \times 10^3$	0.9996	0.003
Reb B	$Y = 3.52 \times 10^5 X - 2.94 \times 10^2$	0.9993	0.002
SVB	$Y = 3.11 \times 10^5 X + 4.58 \times 10^2$	0.9993	0.003

Table 1.B

	Sample A		Sample B		Sample D	
	Reb N	Reb M	Reb N	Reb M	Reb N	Reb M
Mean (native) (%, w/w)	0.00%	0.00%	0.060%	0.272%	0.769%	1.845%
Spike level (low) (%, w/w)	0.17%	0.22%	0.17%	0.22%	0.24%	0.30%
Mean Recovery (%)	95.9%	102.2%	106.5%	111.8%	99.9%	102.0%
Spike level (high) (%, w/w)	1.71%	2.17%	1.74%	2.21%	2.36%	3.00%
Mean Recovery (%)	105.3%	99.7%	102.9%	106.3%	98.2%	99.1%

Table 1. *(A) Linear relationship between the peak area and the concentration and the estimated limit of quantification. (B) Spiking recovery results on three sample matrices for steviol glycosides.*

3) Resolutions on multiple columns and on a sub-2 micron column

Table 2. *Resolutions for LC-UV separation of steviol glycosides on various HSS T3 Columns and a sub-2 micron particle column.*

	Reb E	Reb O	Reb D	Reb N	Reb M	Reb I	Reb A	SV	Iso Reb A	Reb F	Reb C	Dul A	Rub	Reb B	SVB
Column	**XSelect Premier HSS T3 Column (2.5 µm, 4.6 x 150 mm)**														
Resolution (USP HH)															
Mean (n=5)	-	2.17	1.94	2.93	4.26	25.77	3.89	1.63	2.28	4.94	3.56	2.91	9.24	8.53	*
RSD (%)	-	0.21	0.05	0.2	0.09	0.15	0.15	0.18	0.16	0.13	0.15	0.16	0.1	0.11	*
Column	**XSelect Premier HSS T3 VanGuard FIT Column (2.5 µm, 4.6 x 150 mm)**														
Resolution (USP HH)															
Mean (n=6)	-	2.01	1.99	2.86	4.24	25.65	3.94	1.69	2.25	4.99	3.60	2.99	9.46	8.61	*
RSD (%)	-	1.35	0.53	1.34	0.29	0.75	0.69	0.68	0.97	0.65	0.80	0.65	0.57	0.73	*
Column	**XSelect HSS T3 Column (2.5 µm, 4.6 x 150 mm)**														
Resolution (USP HH)															
Mean (n=5)	-	1.90	1.89	2.56	4.10	23.81	3.65	1.51	2.18	4.64	3.39	2.71	8.83	8.35	2.70
RSD (%)	-	0.44	0.51	0.32	0.37	0.2	0.07	0.18	0.16	0.1	0.19	0.09	0.1	0.08	0.23
Column	**ACQUITY UPLC HSS T3 Column (1.8 µm, 3 mm x 150 mm)****														
Resolution (USP HH)															
Mean (n=6)	-	2.39	2.19	3.33	4.77	29.45	4.57	2.01	2.57	5.84	4.13	3.55	11.1	9.91	*
RSD (%)	-	0.18	0.33	0.13	0.27	0.13	0.12	0.06	0.14	0.11	0.07	0.13	0.06	0.05	

Note:
* Resolution larger than 2.7 was obtained between Reb B and SVB. The resolution was not shown because the original number was calculated incorrectly (due to a small peak between Reb B and SVB).
** Results obtained under conditions for a sub-2 micron particle-size column.

CONCLUSION

- **The JECFA LC-UV analysis of steviol glycosides has been improved. The Resolution for the critical pair (Reb A/SV) were:**
  - **Rs ≥ 1.5 on XSelect Premier HSS T3 Columns (2.5 µm, 4.6 mm X 150 mm).**
  - **Rs ≥ 2.0 on ACQUITY UPLC HSS T3 Columns (1.8 µm, 3.0 mm X 150 mm).**
- **The enhanced approach to method development in the ICH Q14 Analytical Procedure Development guideline was adopted in the method optimization.**
- **Excellent analytical performance in linearity, sensitivity, accuracy, precision, and robustness has also been demonstrated.**
- **This developed method could be a useful alternative method for the analysis of steviol glycosides.**

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