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Comparative Studies on the Assay Methods of *Stevia* Sweeteners

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스테비아 감미성분의 정량법에 관한비교

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Abstract

Analytical methods on *Stevia* sweeteners are compared for their reproducibilities and recoveries. It is possible to separate stevioside, rebaudioside A, rebaudioside C, and dulcoside A through HPLC analysis. Steviolbioside, in addition to above 4 *Stevia* sweeteners, is detected through TLC scanner and TLC-FID assays. C.V.s on stevioside and rebaudioside A in HPLC analysis are 1.39 and 4.89%, which shows outstanding reproducibilities of this method. The recoveries of stevioside in HPLC, TLC scanner, and TLC-FID analyses are 97.7 89.4, and 97.3%. The recoveries of rebaudioside A in HPLC, TLC scanner, and TLC-FID assays are 90.8, 90.1, and 75.8%. Total content of *Stevia* sweeteners in 8 strains tested, ranges from 5 to 17% as dry weight basis.

Introduction

Stevia rebaudiana Bertoni is a perennial plant of the composite family native to Paraguay. Its leaves contain sweet principles such as stevioside and rebaudioside A.

Until now, quantitative analyses of *Stevia* sweeteners have been done with TLC (Thin Layer Chromatography)[1,2] TLC-FID (Thin Layer Chromatography Flame Ionization Detector[3], and gas chromatography[4]. TLC-FID determination has been routinely used for the determination of *Stevia* sweeteners in this country.

Recently, determinations of *Stevia* sweeteners through HPLC (High Performance Liquid Chromatography)[5,6] and TLC scanner[7] have been tried.

The major goal of this study is to establish proper and easy assay methods on *Stevia* sweeteners. To achieve this aim, HPLC and TLC scanner methods are compared with the conventional TLC-FID method and the results are analyzed.

Materials and Methods

Standard samples of *Stevia* sweeteners

Stevioside, rebaudioside C, rebaudioside D, rebaudioside E, and dulcoside A were kindly donated by Dr. Masaru Kobayashi of Hokkaido University and Dr. Osamu Tanaka of Hiroshima University. Rebaudioside A and steviolbioside have been stocked in FRI.

Analytical instruments

HPLC of Waters Associates, Iatroscan TH 10 TLC-FID analyzer of Iatron Company, and TLC scanner CS-920 of Shimadzu Company were used.

Sample preparation for assay

Four *grams* of dried and mashed *Stevia* leaves was extracted by 50 *ml* of 85% methanol for 2 hours at 45 °C with vigorous shaking. And then, it was further extracted for 15 *hours* at 20 °C with shaking. After 4 *hours* of fixing, it was centrifuged at 13,000 *g* for 10 *minutes* and the supernatant was used for analytical purpose.

Operating conditions for the instruments

A. HPLC

Column: Carbohydrate Analysis Column (Waters, 30 × 0.4 *cm* I.D.)
Eluant: Acetonitrile:water (80:20)
Flow rate: 1.5 *ml/min*
Detector: Refractive index detector (attenuator: RI 8x)
Chart speed: 5 *mm/min*
Injection: 10 μl (5-100 μg)

B. TLC scanner

Plate: 20 × 20 *cm* glass plate
Stationary phase: Kiesel gel 60G (Merck)
Mobile phase: Ethylacetate:isopropanol:water (68:21:11)
Coloring: 10N H_2SO_4
Scanning wavelength: 450 *nm*
Chart speed: 10 *mm/min*
Signal output: 100 *mv*
Spotting: 40 μl (20-400 μg)

C. TLC-FID

Flow rate: H_2 160 *ml/min*
Air 2,000 *ml/min*
Stationary phase: Chromarod-SII (Iatron Co.)
Mobile phase: Chloroform:methanol:water (6.5:3:1, lower layer)
Developing time: 90 *min*
Chart speed: 120 *mm/min*
Recorder range: 100 mv f.s for chromatogram
200 mv f.s for integral curve
Spotting: 2 μl (1-20 μg)

Reproducibility test

Coefficient of variability was used as a measure of reproducibility of the analytical method. Content of stevioside and rebaudioside A was measured 15 *times* in each method.

$$\text{Coefficient of variability } (C.V.) = \frac{s}{\bar{x}} \times 100, \%$$

where, s: standard deviation
$\bar{x}$: mean

Recovery test

Three *mg* of standard sample of stevioside and rebaudioside A was added to 1 *ml* of assay solution simultaneously (treatment). Content of stevioside and rebaudioside A in the treatment was compared with that in assay solution without added standard sample (control).

$$\text{Recovery} = \frac{\text{content in treatment-content in control}}{3\ mg/ml} \times 100, \%$$

Results and Discussion

Chromatograms of *Stevia* sweeteners in HPLC, TLC scanner, and TLC-FID analyses are shown in Fig. 1. Dulcoside A and rebaudioside C form brown spots on the TLC plate, whereas, stevioside, rebaudioside A, and steviolbioside show black spots. As the mono and disaccharides such as glucose and sucrose show no or less mobilities, it is possible to ignore interferences of such compounds in the scanning. One unknown spot which has R_f value of 0.80 is found like the result of Sakamoto *et al.*[8] The photograph of the TLC plate developed with some strains of *Stevia rebaudiana* Bertoni is shown in Fig. 2. In the case of HPLC determination, 2 unknown peaks appear in a few strains. They are presumed as the derivatives of *Stevia* sweeteners or saccharides present in the leaves of *Stevia*.

Since their chemical structures were identified by some researchers[9,10], dulcoside A and rebaudioside C have been known as two of minor glycosides in *Stevia*. However, quantitative analytical methods on them have not been published yet except dulcoside A is separated as a small peak in TLC-FID assay[3].

Among the diterpenoid glycosides known to be present in *Stevia*[6,11], rebaudioside B, rebaudioside D, and rebaudioside E are unable to be separated under the operating conditions of the instruments in this research. More efforts are needed to separate and determine them.

Standard curves of *Stevia* sweeteners in HPLC, TLC scanner, and TLC-FID assays are shown in Fig. 3, Fig. 4, and Fig. 5. A series of concentration (0.5-10 *mg/ml*) of *Stevia* sweeteners was made and used for the preparation of the standard curve.

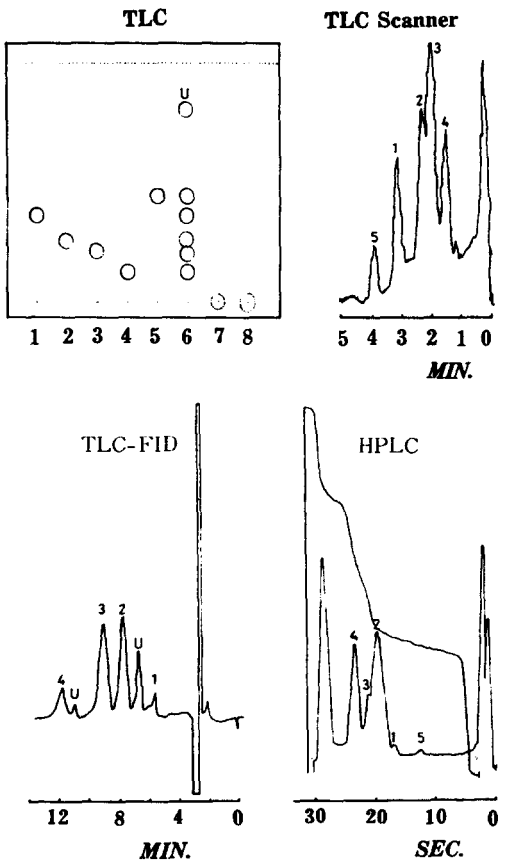

Fig. 1. Chromatograms of *Stevia* sweeteners in HPLC, TLC scanner, and TLC-FID assays
1:Dulcoside A, 2:Stevioside, 3:Rebaudioside C, 4:Rebaudioside A, 5:Steviolbioside, 6:Extract of *Stevia*, 7:Sucrose, 8:Glucose, U:Unknown

HPLC;
Eluant—acetonitrile:water (80:20)
Retention time (min)—1:5.6, 2:7.6, 3:9.0
4:11.7

TLC scanner;
Solvent system—ethylacetate:isopropanol:
H_2O (68:21:11)

R_f—1:0.35, 2:0.26, 3:0.21, 4:0.13, 5:0.44
U:0.80

TLC-FID;
Solvent system-chloroform:methanol:water
(6.5:3:1, lower layer)
Retention time (*sec*)—1:17, 2:20, 3:21, 4:24
5:12

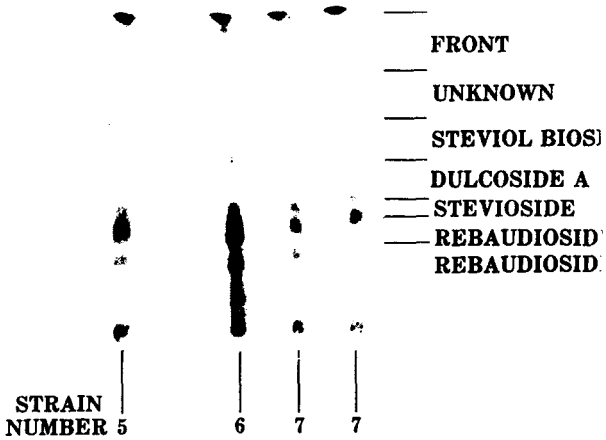

Fig. 2. The photograph of the TLC plate

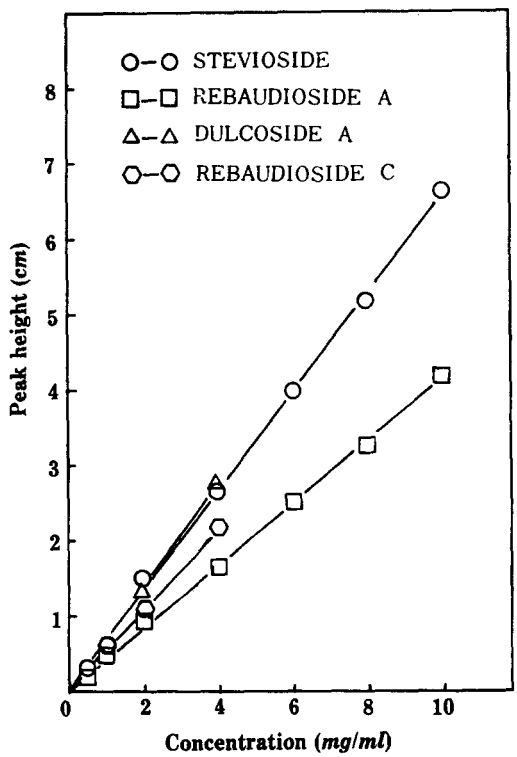

Fig. 3. Standard curves of *Stevia* sweeteners in HPLC assay

The content of 2 major *Stevia* sweeteners, stevioside and rebaudioside A, is measured from the assay solution to compare the mean values and reproducibilities of the analytical methods (Table 1). Like represented in Table 1, the mean values of stevioside in HPLC and TLC-FID assays are similar, however, that in TLC scanner

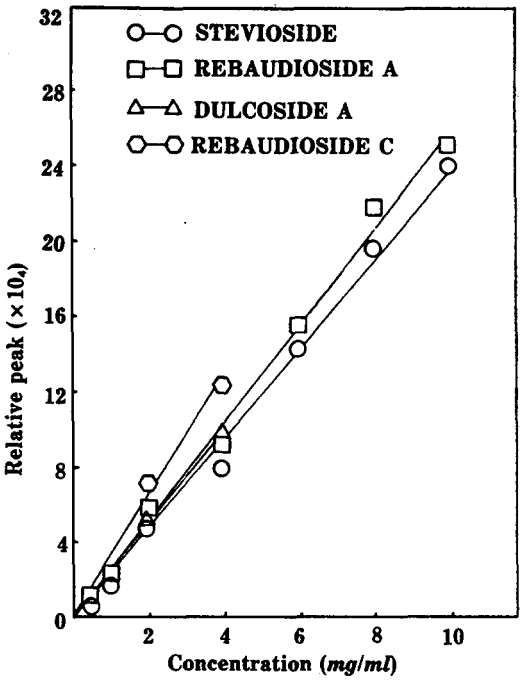

Fig. 4. Standard curves of *Stevia* sweeteners in TLC scanner assay

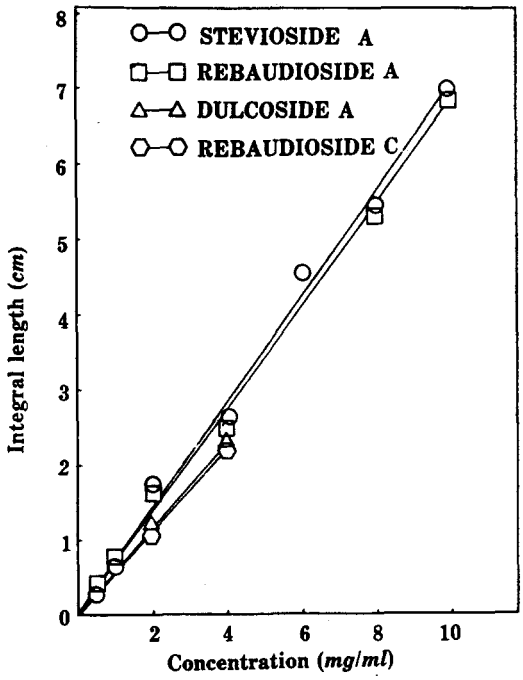

Fig. 5. Standard curves of *Stevia* sweeteners in TLC-FID assay

Table 1. Comparison on the assay methods of *Stevia* sweeteners

	Stevioside, %*			Rebaudioside A, %		
	HPLC	TLC scanner	TLC-FID	HPLC	TLC scanner	TLC-FID
	6.89	9.00	6.45	4.13	4.76	3.24
	6.83	8.88	5.59	3.75	4.38	2.83
	6.96	8.58	7.34	4.03	3.96	3.74
	6.96	7.14	7.20	4.19	3.91	3.36
	6.88	5.68	5.85	3.84	3.89	3.50
	6.71	6.94	7.24	4.03	3.39	3.81
	7.10	6.23	6.73	4.03	4.09	3.28
	6.89	7.18	6.65	3.75	3.88	3.43
	6.91	7.90	6.08	4.13	3.72	3.61
	6.80	6.28	7.78	3.88	4.50	4.33
	6.96	8.17	8.84	4.03	4.13	4.29
	7.04	9.16	5.95	4.34	4.06	3.53
	6.89	8.38	8.08	4.34	3.71	3.49
	7.00	8.08	7.84	4.34	3.85	4.04
	6.91	8.46	7.60	4.19	3.88	3.80
x	6.92	7.74	7.01	4.07	4.01	3.63
Standard deviation	0.096	1.097	0.933	0.199	0.339	0.404
C.V., %	1.39	14.17	13.31	4.89	8.45	11.13

%*: As dry weight basis

analysis is slightly higher than those in 2 other methods. This can be attributed to the fact that content of rebaudioside C is added to that of stevioside on scanning, in many instances, according to the condition of developed plate. Interfering substances such as pigments, are likely to cause bad state of plate. In fact, discriminative determination on stevioside and rebaudioside C is only possible in 2 strains of *Stevia rebaudiana* Bertoni in TLC scanner assay (Table 3). The mean values of rebaudioside A in HPLC and TLC scanner assays are alike, with slightly lower value in TLC-FID assay.

As shown in Table 1, C.V.s on stevioside in HPLC and TLC scanner, and TLC-FID assays are 1.39, 14.17, and 13.31%. C.V. on rebaudioside A in HPLC analysis is 4.89%, which is lower than 8.45% in TLC scanner assay and 11.13% in TLC-FID assay. As C.V.s in HPLC analysis are much lower than those in other methods, it is evident to say HPLC method has excellent reproducibilities in the determinations of stevioside and rebaudioside A. C.V.s on rebaudioside C and dulcoside A are 9.25 and 35.30% in HPLC assay.

Table 2 describes the results of the recovery tests on stevioside and rebaudioside A in HPLC, TLC scanner, and TLC-FID assays. Till now, there has been no report on the recovery test about *Stevia* sweeteners except that reported by Sugisawa *et al*.[12] They said that the recovery on stevioside in TLC-FID assay reached from 91.3 to 93.6%. As mentioned in Table 2, the recoveries on stevioside in HPLC, TLC scanner, and TLC-FID analyses are 97.7, 89.4, and 97.3%. In this research, the recovery on stevioside in TLC-FID assay is slightly higher than the value stated by Sugisawa *et al*. The recoveries on rebaudioside A are generally lower than those on stevioside.

Table 2. Recovery tests on stevioside and rebaudioside A

Assay method	Recovery, %	
	Stevioside	Rebaudioside A
HPLC	97.7	90.8
TLC scanner	89.4	90.1
TLC-FID	97.3	75.8

Content of *Stevia* sweeteners in 8 strains of *Stevia rebaudiana* Bertoni was measured by using HPLC and TLC scanner (Table 3). Relatively high content of rebaudioside C is found in some strains. There is conspicuous difference in content of stevioside between HPLC and TLC scanner assays in some strains, which is likely to be due to the fact that content of rebaudioside C is included in that of stevioside on scanning, in many cases, in TLC scanner assay.

As described above, HPLC analysis on *Stevia* sweeteners is superior in reproducibility and recovery than TLC scanner and conventional TLC-FID assays. However, if 2 dimensional development is used, TLC scanner assay will be expected to give more correct and reliable results.

Table 3. Content of stevioside, rebaudioside A, dulcoside A, and rebaudioside C as dry weight basis in 8 strains of *Stevia rebaudiana* Bertoni

Strain number	Stevioside, %		Rebaudioside A, %		Dulcoside A, %		Rebaudioside C, %	
	HPLC	TLC scanner	HPLC	TLC scanner	HPLC	TLC scanner	HPLC	TLC scanner
1	9.85	11.02	3.78	4.50	0.36	0.90	2.11	-
2	7.71	7.30	3.43	3.06	0.36	0.86	1.94	-
3	6.74	6.47	4.56	4.37	0.36	0.69	1.89	-
4	7.66	7.60	5.08	5.00	0.46	0.31	1.85	-
5	2.01	6.73	1.12	1.49	0.36	1.37	3.87	-
6	4.45	11.79	2.02	3.24	0.48	2.10	5.34	-
7	2.22	2.28	0.95	1.09	0.37	1.07	2.38	2.51
8	2.41	2.42	1.42	1.64	0.25	0.43	0.72	0.30

요 약

HPLC법으로는 stevioside, rebaudioside A, dulcoside A, rebaudioside C의 분리 정량이 가능하였고 TLC scanner법과 TLC-FID법상으로는 위의 4성분 외에 steviolbioside가 분리정량 되었다. HPLC 법에서 stevioside와 rebaudioside A에 대한 변이 계수는 1.39%와 4.89%로서 HPLC법의 높은 재현성을 보여주고 있다.

HPLC, TLC scanner, TLC-FID법에서 stevioside의 회수율은 97.7, 89.4, 97.3%이고 rebaudioside A의 회수율은 90.8, 90.1, 75.8%였다. 분석시료 8종중 *Stevia* 감미성분의 전체 함량은 5%에서 17% 사이였다.

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