Subcritical Fluid Extraction of Stevia Sweeteners from *Stevia rebaudiana*

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Abstract

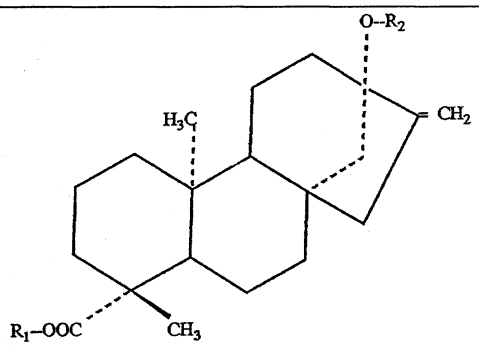
In this paper, the extraction of steviol glycosides in *Stevia rebaudiana*, including stevioside, rebaudioside A, rebaudioside C, and dulcoside A by subcritical fluid extraction (SubFE) is investigated. A simple, efficient SubFE method is developed. The extraction conditions (extraction phase composition, extraction time, etc.) are optimized. An extraction efficiency of more than 88% is obtained using methanol as a modifier. Determination of stevioside in the leaves of *Stevia rebaudiana* is performed by using the SubFE method developed and capillary electrophoresis.

Introduction

The plant *Stevia rebaudiana* Bertoni (Compositae) has been widely cultivated in the world for the sweet diterpene glycosides that are mainly contained in its leaves. Stevia sweeteners, the extraction products, are commonly used as nonnutritive and high-intensity sweeteners in beverages, foods, and medicines. There are eight steviol glycosides found in plants in which stevioside (SS) is the major constituent and rebaudioside A (RA), rebaudioside C (RC), and dulcoside A (DA) are the other main constituents. Their structures are shown in Figure 1 (1). Numerous methods for stevia extraction have been described. Most refining processes appear to involve aqueous or alcohol extraction followed by precipitation and coagulation with filtration and a final cleanup on exchange resins before crystallization and drying (2). These extraction techniques provide good results, but all of them are long and tedious and require large quantities of solvents. Compared with liquid extraction, supercritical fluid extraction (SFE) and subcritical fluid extraction (SubFE) show some advantages, including: (*a*) they are rapid, simple, and less expensive in terms of solvent cost, (*b*) variation of the extraction conditions can be introduced by changing the pressure, temperature, and nature of the fluids used, and (*c*) they generate extracts that are ready for analysis (e.g., coupled on- or off-line with chromatographic techniques without additional concentration or class fractionation while producing little or no laboratory waste). At present, it has been gaining popularity as an analytical extraction method for sample preparation prior to chromatographic analysis (3–8).

As for the extraction fluids, CO_2 has been the most widely used so far, but pure CO_2 is not effective for extracting very polar molecules, and organic modifiers are usually added in the extraction fluid. There are three different procedures for generating modified CO_2. Among them, the dual pumps system is more useful for dynamic SFE, and operation is relatively easy (9).

This paper describes an efficient and simple method for extracting stevia glycosides by SubFE (CO_2 + polar modifier) using a homemade SFE instrument. It was evaluated by studying the effect of different extraction conditions (pressure, temperature, and nature of the extraction phase) on recoveries. The extracts were determined by capillary electrophoresis (CE). The results were compared with other liquid extraction methods.



Name	R_1*	R_2*
Dulcoside A	glc	glc-rham
Stevioside	glc	glc-glc
Rebaudicoside C	glc	glc-rham \ glc
Rebaudicoside A	glc	glc-glc \glc

* glc = α-D-glucopyranosyl; rham = β-L-rhamnopyranosyl.

Figure 1. Names and structures of steviol glycosides.

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Experimental

Reagents and materials

Stevioside standard was purchased from Wako (Osaka, Japan). Leaves of *Stevia rebaudiana* were provided by Professor Liu Kailu (Beijing Research Institute of Chemical Engineering and Metallurgy, Beijing, P.R. China).

Acetonitrile (HPLC grade) was purchased from BDH (Poole, England). Methanol was from Fisher Scientific (Fair Lawn, NJ). Other chemicals were supplied by Fluka (Buchs, Switzerland). Deionized water was obtained from a Milli-Q system (Millipore, Bedford, MA). All the chemicals used were analytical grade. SFE and SubFE experiments were carried out using high-purity (CP-grade) CO_2 (BOC, London, England).

SFE and SubFE instrumentation

All extractions were performed using the homemade SFE system modified from previous literature (10). The HPLC pumps (Shimadzu LC-6A, Kyoto, Japan) were used to deliver CO_2 and the organic modifiers, which were mixed together through a $^1/_{16}$-inch T-tube before the extraction chamber. The composition of the mixed fluids was calculated from the flow rates of the modifiers and CO_2, and the flow rates of modifers were adjusted by the HPLC pump, which delivered the modifiers in the range of 0.1–0.3 mL/min. The flow rates of CO_2 were controlled by the restrictor and determined with a soap-bubble meter (Supelco Optiflow 520 digital flowmeter, Bellefonte, PA). A metal restrictor, the VR100 variable restrictor (CCS Instrument Systems, West Grove, PA) was used in this extraction system because the linear restrictor, which was made from a fused-silica capillary, was often blocked during extraction with polar solvents (11). An extraction cell was made from an empty stainless steel HPLC column (50 × 4.6-mm i.d.) (Phase Separations, Clwyd, United Kingdom), which was housed in a model 5790A gas chromatography (GC) oven (Hewlett-Packard, Wilmington, DE). The pressure was controlled by the pumps, and the temperature was controlled by the oven.

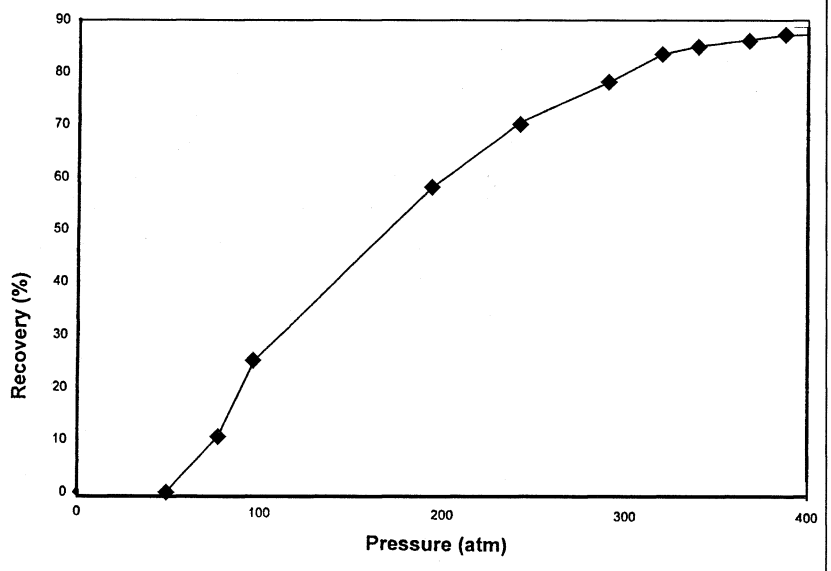

Figure 2. Influence of extraction pressure on stevioside recovery. Sample, 200 mg glass wool spiked with 0.5 mg stevioside; extraction time, 90 min; extraction temperature, 40°C; extraction fluid, CO_2–CH_3OH (80:20, v/v); extract collection in methanol.

CE system

CE was carried out on a commercial and a laboratory-built CE system. For the laboratory-built CE system, a Spellman (Plainview, NY) 30-kV power supply was used. A fused-silica capillary tube (50 cm effective length, 50-mm i.d.) (Polymicro Technologies, Phoenix, AZ) was used as the separation column. The peaks were detected by a Micro-UVis20 detector (Carlo Erba, Milan, Italy) with the wavelength set at 210 nm. The window for the on-column detection cell was made by removing a small section of the polyimide coating on the fused-silica capillary. Data processing was performed on a Shimadzu (Kyoto, Japan) Chromatopac C-R6A instrument, and samples were injected into the capillary by gravity feed with an injection time of 20 s and injection height of 10 cm. The commercial system was an HP^{3D} CE system (Hewlett-Packard, Wurzburg, Germany) equipped with the same capillary. Samples were injected into the capillary by pressure (30 mbar, 20 s). The separation conditions used were similiar to those of our previous work (12): an applied voltage of 16.5 kV and a buffer solution containing 50mM sodium tetraborate (pH 9.3) and 55% acetonitrile (acetonitrile-to-buffer ratio, 55:45, v/v).

SFE procedure

Appropriate amounts of accurately weighed dry leaves and stevioside standard were used throughout the work. The dry leaves were totally ground before extraction. Stevioside standard solution (500 ppm) was prepared with methanol. The standard solution (1 mL) was spiked into the glass wool, and the solvents were evaporated before extraction. The samples (including dry leaves and the spiked ones) were manually placed into the extraction cell. Extracts were collected in 3 mL of organic solvents in a 5 mL volumeric flask. The restrictor was immersed below the surface of the collecting solvent, and the flask was covered with a piece of Nescofilm (Nippon Shoji, Kaisha, Osaka, Japan). Collection was performed at room temperature. After collection, when acetonitrile and water were used as the modifiers, the extracts were diluted with the same modifier in a 10-mL volumetric flask, filtered, and then analyzed by CE. When methanol was used as the organic modifier, the extract solutions were evaporated, and the residues were dissolved in the solution (acetonitrile–water, 80:20, v/v) and analyzed by CE.

Liquid extraction of stevioside

Dried leaves (2.5 g) were extracted with hot methanol for 7 h, and the methanol solution was concentrated to dryness. A suspension of the residue in water was washed with ether and then extracted with butanol. The organic phase was evaporated, and the residue was recrystallized from methanol, giving stevioside. Then the

Table I. Effect of Modifier Concentrations on Extraction Efficiency*

Concentration (%)	5	10	15	20	25
CH_3OH	16.4	58.3	75.6	88.4	88.7
CH_3CN–H_2O (80:20, v/v)	8.3	42.8	66.4	81.6	85.2

*Stevioside spiked samples, five replicates.

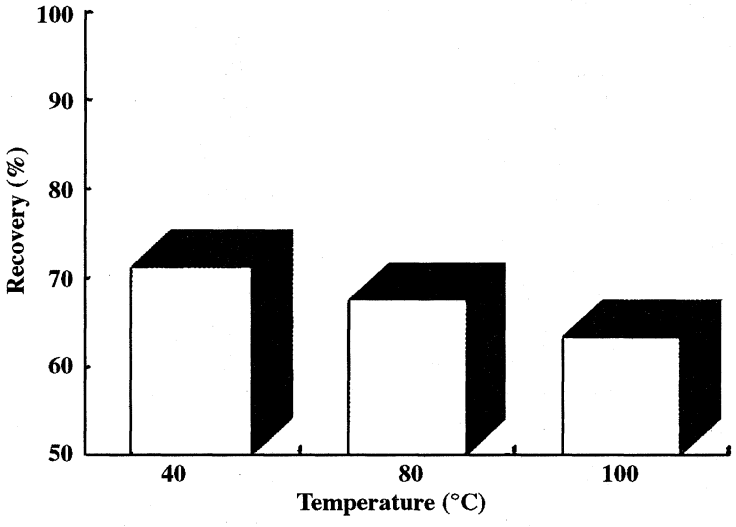

Figure 3. Influence of temperature on stevioside recovery. Sample, 200 mg glass wool spiked with 0.5 mg stevioside; pressure, 368 atm; extraction fluid, CO_2–CH_3OH (80:20, v/v); other conditions as in Figure 2.

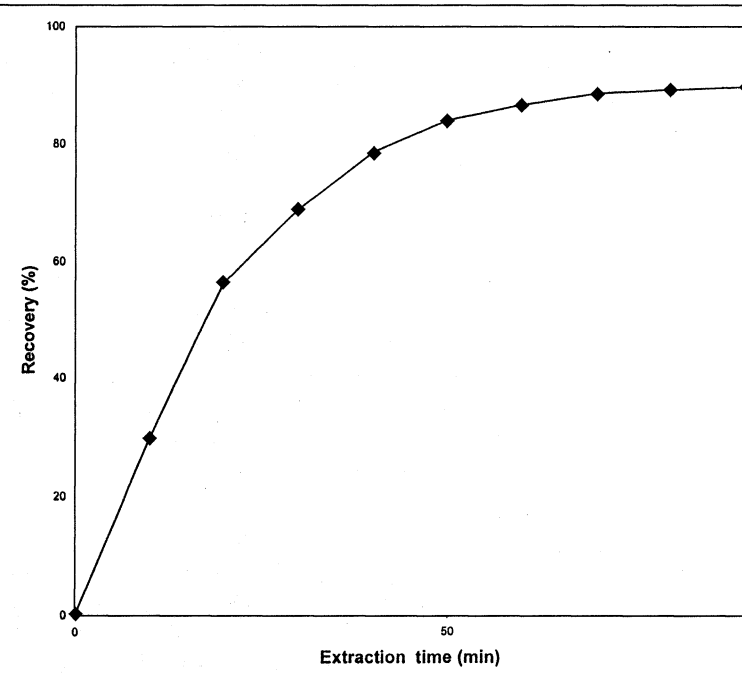

Figure 4. Influence of extraction time on stevioside recovery. Sample, 200 mg glass wool spiked with 0.5 mg stevioside; pressure, 368 atm; extraction fluid, CO_2–CH_3OH (80:20, v/v); other conditions as in Figure 2.

recrystallized stevioside was dissolved in the solution (acetonitrile–water, 80:20, v/v) and analyzed by CE.

Results and Discussion

The solubilities of solutes in supercritical and subcritical fluids depend on their polarity, the density of the fluid, and the temperature. Pure supercritcal CO_2 is a solvent of low polarity; its effectiveness as an extraction fluid is limited to relatively nonpolar and moderately polar solutes and not suitable for more polar ones, such as the sweet diterpene glycosides studied in this paper. This problem can be alleviated by the addition of small amounts of a polar modifier in carbon dioxide. Therefore, CO_2 with organic modifiers was chosen as the extraction system. The density is a function of temperature and pressure. The higher the temperature, the higher the pressure that is generally required to achieve a similar fluid density. Consequently, it is advantageous to work at a temperature close to the critical temperature. However, by increasing the temperature, the diffusivities of the solutes in the supercritical fluid increase, and thus the rate of extraction may be increased. Therefore, the extraction behavior was studied by varying the pressure and temperature and the polarity of the extracting phase.

Stevia sweeteners mainly contain four steviol glycosides (i.e., SS, RA, RC, and DA). Stevioside was chosen as a model substance because of its presence in large amounts in the stevia sweeteners and the similar polarity of the steviol glycosides. It was expected that other stevia glycosides would also be extracted when stevioside was quantitatively extracted.

Optimization of SFE conditions

The addition of polar modifiers in the CO_2 fluid tends to improve the extraction yield for polar compounds. The modifier decreased the adsorption of polar solutes on the surface of polar matrices and might improve the swelling of the matrix to accelerate solute diffusion out of the matrix (13). Modifier selection was based on their different polarities. Though many different modifiers have been employed in previous studies, methanol remained the most popular one. In our experiments, the stevioside standard was extracted successively from the spiked samples with increasing concentrations of methanol at a constant pressure and temperature. Table I shows that the extraction efficiencies increased up to 88.4% for 20% of methanol (volume percent), and further increase in polarity did not yield significantly more stevioside. Therefore, 20% methanol-modified CO_2 was chosen as the extraction phase.

Another modifier system employed in this extraction was acetonitrile–water (80:20, v/v), which was used as the sample solvent in subsequent CE analysis. This extraction system was convenient to use because the

extracted solutes could be analyzed directly by CE without the need to evaporate the solvent for collection and then redissolve the residues into the sample solvent. However, the extraction result showed that this system was not as good as the methanol-modified extraction fluid in terms of ruggedness. The reason is that, although water acted as a swelling agent in the system, high concentrations of water sometimes caused restrictor plugging and resulted in unreliable measurements. Other reasons for the low recovery could be the poor solubility of acetonitrile–water in CO_2 under these conditions and the inefficient trapping procedure.

The fluid pressure was the main parameter in the extraction system. The increase in pressure at a constant temperature and fluid composition resulted in an increase in the fluid density and hence the solubility of the solutes. This was demonstrated for the extraction of stevioside from glass wool in Figure 2. It was shown that the extraction efficiency increased significantly with increasing pressure and a constant temperature (40°C) and extraction fluid composition (methanol–water, 80:20). No stevioside was extracted below a certain pressure. The maximum recovery was about 88% at a pressure of 368 atm for 90 min. Above this pressure, increasing the pressure further did not improve the recoveries.

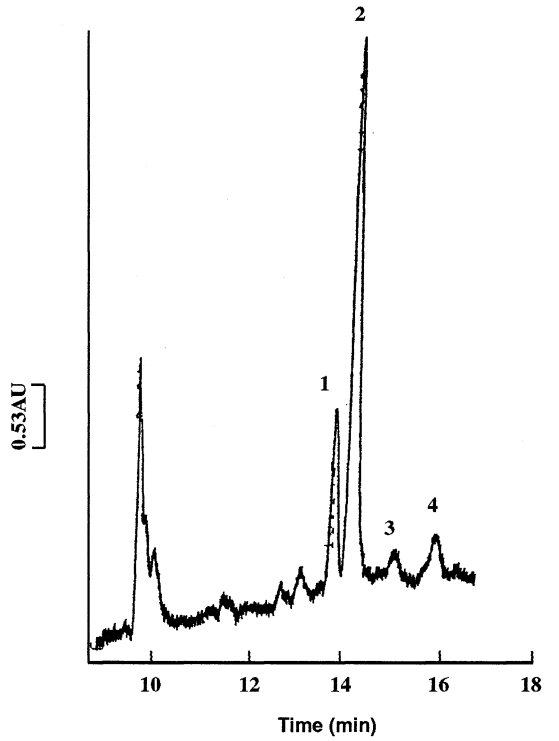

Figure 5. Capillary electropherogram of plant extract by SubFE under optimal conditions. Sample, 12 mg dry leaves of *Stevia rebaudiana*. Buffer, 50mM sodium tetraborate (pH 9.3)–acetonitrile (55:45, v/v); UV detection, 210 nm; voltage, 16.5 kV; temperature, ambient. Peaks: 1, rebaudicoside A; 2, stevioside; 3, dulcoside A; 4, rebaudicoside C.

Table II. Determination of Stevioside in the Leaves of *Stevia rebaudiana*

	SubFE (5 replicates)	Liquid extraction (5 replicates)
Analyzed by CE (%)	13.7 ± 5.8	13.1 ± 9.3

The influence of modifiers on recovery was more pronounced at lower temperatures, mainly because of the higher densities at lower temperatures. In addition, at a high pressure (368 atm), temperature had relatively little influence on extraction efficiencies because temperature variations at high pressure did not significantly affect the density of the fluids, whereas at a low pressure (242 atm), efficiencies decreased with an increase in temperature (see Figure 3). Therefore, 40°C was chosen as the extraction temperature. At this temperature, the modified CO_2 was not at the supercritical state but at the subcritical state (14). Hence the subcritical fluid instead of the supercritcal fluid was used in these experiments.

Extraction and analysis of stevia sweeteners from real samples

The stevioside spiked samples were extracted by SubFE with 20% MeOH-modified CO_2; all the other conditions were the same as before. The recoveries obtained for various SubFE extraction times are shown in Figure 4. It was noted that the percentage recoveries increased with the extraction time up to about 90 min (including 10 min of static extraction), after which no significant increase in recoveries was observed. Therefore, a 90-min extraction time was chosen. Five SubFE extractions were performed to obtain a mean recovery of 88.4% with a relative standard deviation (RSD) of 3.2%. There were some losses in the extraction procedures, such as some possible losses in the collection and evaporation procedures, which made the recoveries less than 100%. Because the results of extracting stevioside were reproducible, the same approach was adopted for the analysis of real samples (the dry leaves), and the extraction results are shown in Table II and Figure 5. For the extraction of the dry leaves, it was found to be better to include a pretreatment step by using pure CO_2 to remove the nonpolar compounds in the plant samples. After using CO_2 to extract the plant samples for 40 min, modified CO_2 was used to extract the stevia sweeteners.

Stevia sweeteners were also extracted by liquid extraction as described in the Experimental section. The stevioside contents are also shown in Table II. Comparing the results obtained by SubFE and liquid extraction, it was found that good agreement was obtained, but SubFE had the advantages of being fast and easy and consumed less solvents.

All the extracts were analyzed by CE, as described in our previous study (12). Compared with HPLC, CE was simple, required only small amounts of samples, and was suitable for the separation of real samples, whereas the column used in the HPLC system was easily contaminated (12). This coupled SubFE–CE method could be widely applied in the determination of stevia sweeteners in various samples.

Conclusion

In this study, the extraction of stevia sweeteners by SubFE was successfully demonstrated. Optimum conditions for the extraction of the stevia glycosides were obtained. SubFE was easy to perform and fast. Furthermore, the combination of SubFE with CE was a very attractive method for the extraction, separation,

and determination of stevia sweeteners and related compounds in plant samples.

Acknowledgment

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References

1. J.R. Hanson and B.H. De Oliveira. Stevioside and related sweet diterpenoid glycosides. *Nat. Prod. Rep.* **10:** 301–309 (1993).
2. K.C. Phillips. *Developments in Sweeteners-3.* T.H. Grenby, Ed. Elsevier Applied Science, London & New York, 1987, Ch.1, p.1.
3. S. Bowadt and S.B. Hawthrone. Supercritical fluid extraction in environmental analysis. *J. Chromatogr.* **703:** 549–71, (1995).
4. P.Edder, C. Staub, J.L. Veuthey, I. Pierroz, and W. Haerdi. Subcritical fluid extraction of opiates in hair of drug addicts. *J. Chromatogr.* **658:** 75–86 (1994).
5. K. Li and S.F.Y. Li. Determination of alkyllead and alkyltin compounds in solid samples by supercritical fluid extration and micellar electrokinetic capillary chromatography. *J. Chromatogr. Sci.* **33:** 309–15 (1995).
6. K. Li, C.P. Ong, and S.F.Y. Li. Systematic multivariate optimization of supercritical fluid extraction. *J. Chromatogr. Sci.* **32:** 53–56 (1994).
7. R.M. Smith and M.D. Burford. Supercritical fluid extraction and gas chromatographic determination of the sesquiterpene lactone parthenolide in the medicinal herb feverfew (*Tanacetum parthenium*). *J. Chromatogr.* **627:** 255–61 (1992).
8. H. Engelhardt, J. Zapp, and P. Kolla. Sample preparation by supercritical fluid extraction in environmental food and polymer analysis. *Chromatographia* **32:** 527–37 (1991).
9. S.B.Hawthrone. *Supercritical Fluid Extraction and Its Use in Chromatographic Sample Preparation.* S.A. Westwood, Blackie Academic & Professional, London & New York, 1993, Ch 2, pp. 50–54.
10. H. Engelhardt and P. Haas. Possibilities and limitations of SFE in the extraction of aflatoxin B1 from food matrices. *J. Chromatogr. Sci.* **31:** 13–19 (1993).
11. B.C. Bunker, D.M. Haland, T.A. Michalske, and W.L. Smith. Kinetics of dissociative chemisorption on strained edge-shared surface defects on dehydroxylated silica. *Surf. Sci.* **222:** 95–118 (1986).
12. J. Liu and S.F.Y. Li. Separation and determination of stevia sweeteners by capillary electrophoresis and high performance liquid chromatography. *J. Liq. Chromatogr.* **18:** 1703–19 (1995).
13. J.L. Langenfield, S.B. Hawthorne, D.J. Miller, and J. Pawliszyn. Role of modifiers for analytical-scale supercritical fluid extraction of environmental samples. *Anal. Chem.* **66:** 909–16 (1994).
14. G. Brunner and W. Hultenschmidt. Fluid mixtures at high pressures, IV. Isothermal phase equilibria in binary mixtures consisting of (methanol + hydrogen or nitrogen or methane or carbon monoxide or carbon dioxide) *J. Chem. Thermodynam.* **19:** 273–91 (1987).

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