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Supercritical CO_2 extraction of glycosides from *Stevia rebaudiana* leaves: Identification and optimization

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ABSTRACT

The aim of this work was to optimize the glycoside composition of *Stevia rebaudiana* leaves using supercritical fluid extraction (SFE). A Box-Behnken statistical design was used to evaluate the effect of various values of pressure (150–350 bar), temperature (40–80 °C) and concentration of ethanol-water mixture (70:30) as co-solvent (0–20%) by CO_2 flow rate of 15 g min^{-1} for 60 min. The most effective variables were co-solvent concentration ($P < 0.005$) and temperature ($P \leq 0.005$). Evaluative criteria for both dependent variables (stevioside and rebaudioside A yields) in the model was assigned maximum. Optimum extraction conditions were elicited as 211 bar, 80 °C and 17.4% which yielded 36.66 mg/g stevioside and 17.79 mg/g rebaudioside A. Total glycosides composition were close to those obtained using conventional water extraction (64.49 mg/g) and a little higher than ethanol extraction (48.60 mg/g) demonstrating challenges for industrial scale application of SFE.

1. Introduction

In 1900, two types of low calorie natural substances: stevioside and rebaudioside A were isolated [1,2] from *Stevia rebaudiana* Bertoni which is being cultivated in continental China, Taiwan, Thailand, Korea, Brazil, and Malaysia and today in Israel, Ukraine, UK, Philippines, Canada, Hawaii, California and all over South America [3]. All diterpene glycosides isolated from *S. rebaudiana* leaves have the same steviol and differ in the content of carbohydrate residues [4]. HPLC methods have also been reported for the quantification of steviol glycosides determining eight glycosides [5–7]. The major diterpene glycosides are stevioside (5–18%), rebaudioside A (2–4%). Stevioside and rebaudioside A have been rated as possessing about 300 times the relative sweetness intensity of 0.4% (w/v) sucrose [8].

S. rebaudiana extracts are used as low-calorie sweetener and medicinal plant [9] in Japan since 1968, and subsequently have been introduced in other countries such as Brazil, Indonesia, Korea, Mexico, Tanzania, Singapore, Thailand, China, USA, and since 1990 Canada [10,11]. *S. rebaudiana* leaves and extracts can be sold as a dietary supplement in USA [10,12]. The extracts have been used for sweetening soft drinks such as diet coke, soju, soy sauce, dried seafood, candies, ice cream, chewing gum, yoghurt, and as well as in toothpaste and mouthwash in Japan, Korea, and Brazil [13,14].

Various studies reported for this supplement provided data to fill the knowledge gaps identified by The Joint FAO/WHO Expert Committee on Food Additives. Elicited data supported the safety of repeated, long-term consumption of steviol glycosides in humans [13,15–17] and was reported to be neither carcinogenic nor mutagenic [10,18–20]. The extracts can be used by normal persons as well as by diabetics [21]. The extract could play an important role in improving antioxidant intake in human diet. Also, obese persons might lose weight by the fact that excessive sugar in the food is replaced by *S. rebaudiana* extract or stevioside. Regular consumption of these compounds decreases the content of sugar, radionuclides, and cholesterol in blood [22], improves cell regeneration and blood coagulation, suppresses neoplastic growth and strengthens blood vessels [15,23–25].

The mentioned two compounds also exhibit choleretic, anti inflammatory and diuretic properties [19] and prevent ulceration in the gastrointestinal tract [4,25], also exhibit anti-hyperglycemic [26], antihypertensive [27,28], anti-tumor, antidiarrheal, immunomodulatory activities [29]. Therefore, stevioside might be valuable not only as a natural sweetener, but also as a chemo preventive agent against chemical carcinogenesis [29,30].

Stevioside is usually determined by hot water leaching or supercritical fluid extraction (SFE) followed by liquid-chromatographic analysis of the extract [31]. SFE employing CO_2 as a medium for extraction is faster than the conventional method yielding no residual solvent in the final extract, since it is a gas under ambient conditions. It benefits from the physical–chemical properties of supercritical CO_2, which possesses a higher diffusivity and lower viscosity than conventional liquid solvents. However, pure CO_2 does

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not have sufficient solvation power for polar stevioside and therefore a polar co-solvent has to be added. Investigated co-solvents were methanol, water, ethanol and mixtures of these solvents [1,11,31,32].

As far as our literature search could ascertain, no systematic optimization study of the supercritical fluid extraction of *S. rebaudiana* leaves existed. Hence, the objective of this study was to optimize the conditions in SFE, using response surface methodology (RSM) and to compare the glycoside contents with that of traditional water and ethanol extractions of *S. rebaudiana* grown for the first time in Turkey.

2. Materials and methods

2.1. Materials

S. rebaudiana leaves were harvested from the plantation fields in Antalya, Turkey. The leaves were dried at room temperature for 5 days and stored at the cold storage room at +4 °C. Before the SFE runs, the plant material was triturated using the Waring laboratory scale blender (the mean size was 500 μm), packed in plastic bags and stored at +4 °C.

2.2. Chemicals

CO_2 (99%) was taken from Habas, Izmir, Turkey. HPLC standards stevioside and rebaudioside A (95%) were kindly donated by Herbal Wise Ltd., Malaysia. Ethanol, acetonitrile and methanol were of HPLC grade and purchased from Merck (Darmstadt, Germany). Nanopure water used in the analysis were prepared by using in-house nanopure water system (Sartorius Arium 611, Sartorius-Stedim, Goettingen, Germany)

2.3. Supercritical (CO_2 with/without co-solvent) extraction

Supercritical CO_2 extraction was carried out at SFE 100 System (Thar Instruments, Inc., UK, 2006). The extractor volume was 100 ml, thus it was filled with about 30 g of ground *S. rebaudiana* leaves. The independent variables were temperature (40, 60, 80 °C), pressure (150, 250, 350 bar) and co-solvent (ethanol-water) ratio (0%, 10%, 20%). After setting the required values according to the experimental design (Box-Behnken), the extracting pressure and temperature were automatically controlled and maintained throughout the system. When both the set pressure and temperature were reached, the extraction was started and continued for 60 min [32]. The total amount of CO_2 consumed was 900 g during dynamic extraction under each condition. Extracts were collected from the separator outlet after releasing CO_2 from the system.

2.4. Solvent extraction

Ground *S. rebaudiana* leaves (100 g) were extracted with both 1000 ml of ethanol and water for two cycles (about 100 min) [33] using a soxhlet (500 ml) apparatus. The extracts were concentrated to dryness at 70 °C in vacuum by Laborato 4001, Heidolph rotary evaporator and subsequently lyophilized.

2.5. HPLC analysis of the extracts and residues

2.5.1. Sample preparation

100 mg of finely ground dried leaves were sonicated with 10 ml methanol (15 min) for four times. All of the clear extracts were combined and evaporated under vacuum. Evaporated extracts were dissolved in 5 ml HPLC grade methanol in order to determine the glycoside composition of the raw material. Extracts were also dissolved in 5 ml methanol and all the sample solutions were passed through 0.45 μm nylon membrane (SRP 15, Machery Nagel; Düren, Germany) filters to remove non-dissolved particles.

2.5.2. HPLC-UV conditions

HPLC-UV analyses were performed on a HPLC equipped with Shimadzu (Tokyo, Japan) SCL-10A VP control unit, LC – 10 AT VP pump, DGU-14A degasser, SPD-10Avp UV dedector and Rhynodyne manual injector. A Cosmosil® 5NH_2-MS (4.6 mm × 150 mm × 5 μm; Nacalai Tesque, Kyoto, Japan) column was used. The mobile phase comprised water (A) and acetonitrile (B). Isocritic elution was performed starting with 20A/80B, changing the composition to 20A/80B in 20 min. Column was equilibrated for 10 min with the initial conditions prior to the next injection. Detection wavelength and flow rate were set to 210 nm, 1 ml min^{-1}, respectively and the column temperature was ambient.

2.5.3. Calibration

Standard stock solutions were prepared in methanol (3000 μg ml^{-1} for 1 and 2). Five additional levels were prepared by dilution of stock solutions (1500, 750, 500, 205 and 125 μg ml^{-1}) with methanol and stored at 4 °C (Table 1).

2.5.4. Precision and accuracy

Intra- and inter-day precisions of the samples were evaluated with four replicates for day 1 and 2, and three replicates for day 3. The determined amounts of 1 and 2 for intra-day precisions were given in Table 1. One-way ANOVA analysis ($F = 0.505$, compound 1; $F = 0.618$, compound 2) showed that there is no significant difference within the results obtained on three days. Recovery experiments were carried out in triplicates with rebaudioside A (2), at only one concentration level. *S. rebaudiana* leaves were spiked with 100 μg rebaudioside A (2) and the determined recovery rate

Table 1
HPLC results for stevioside (1) and rebaudioside A (2).

Retention times (min)	Stevioside (1)		Rebaudioside A (2)	
	7.10		11.39	
	Average (μg/ml)	%RSD	Average (μg/ml)	%RSD
Day 1 ($n = 4$)	2891.29	2.84	1007.42	4.60
Day 2 ($n = 4$)	2984.61	2.07	1089.76	2.30
Day 3 ($n = 38$)	2959.79	2.65	1067.85	2.53
Accuracy (%)	–		103.7	
Calibration formula ($y = ax, a$)	0.000139367		0.000131131	
LOQ (μg/ml)	3.98		3.98	
LOD (μg/ml)	1.19		1.19	
Linearity range (μg/ml)	3000–50		3000–50	
One-way ANOVA ($\alpha = 0.05$)	0.505		0.618	