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EXPERIMENTAL PAPER

Chemical composition of dried *Stevia rebaudiana* Bertoni leaves and effect of ultrasound-assisted extraction on total steviosides content in extract

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Summary

Introduction: Steviol stevioside, which has been used in the production of food products as a low-calorie sweeten, is one of the main glycoside groups in the leaves of the *Stevia* plants. It is useful for human health.

Objective: The main objection of the present study was to find out some major chemical compositions of the dried *Stevia rebaudiana* Bertoni leaves and an effective, affordable, and environmentally friendly method to reach the high extraction yield of total steviosides from them. Therefore, a novel extraction, ultrasound-assisted extraction (UAE), was carried out to extract total steviosides from dried leaves of *S. rebaudiana* plant with ethanol of 70% (*v/v*) as a solvent.

Methods: Some major chemical compositions of the dried *S. rebaudiana* leaves were analyzed according to the AOAC (Association of Official Analytical Chemists) and total steviosides content (TSC) was measured by anthrone-sulphuric acid colorimetric assay with main influencing factors including material/solvent ratio, extraction temperature, and extraction time.

Results: The results referred that some chemical compounds such as protein, lipid, fibre, sugar, etc. existed in this material. The best extraction conditions were the sample/solvent ratio of 1:100 (g/ml), extraction temperature of 75°C, and extraction time of 30 min.

Conclusion: The highest amount of total steviosides content of 8.894 % was obtained at the optimal extraction condition. Consequently, these results demonstrated that the parameters of UAE were applied successfully for producing total glycosides.

Key words: ***chemical composition, extraction, sweetener, total steviosides content, ultrasound***

Słowa kluczowe: ***skład chemiczny, ekstrakcja, substancja słodząca, zawartość sumy stewiozydów, ultradźwięki***

INTRODUCTION

Stevia which is commonly derived from the plant called *Stevia rebaudiana* (Bertoni) is widely recognized as one of food ingredients. The *Stevia* plant is a herbal plant that flourishes well everywhere from mountainous areas to marginal regions, over from temperate areas to tropical regions. The height of *S. rebaudiana* bushes can reach 1 meter. This plant consists of a long-branched root system, a woody branched stem, elliptical leaves, and white flowers. The leaves are ready to harvest after about 3–4 months of cultivation. It is a rich source of nutritional components such as carbohydrates (35.2–61.93%), proteins (9.8–20.4%), fat (1.9–4.34%), ashes (6.3–13.1%), dietary fibres (15.2–18.5%) [1]. In addition, there are a variety of minerals found in this plant such as K, Ca, Na, Mg, Cu, Mn, Fe, and Zn, while the amount of essential and non-essential amino acids outnumbers those highly recommended by FAO and WHO [2].

In addition**,** the extracts from the plant consist of a number of kinds of steviol glycosides, which is a group of extremely sweet compounds isolated and purified from *S. rebaudiana*. Among them, stevioside and rebaudioside A are the two popular kinds of steviol glycosides identified in plant extracts. Importantly, this plant was classified as a natural source of low-calorie sweetener, due to the fact it can create a low energy value and an intensively sweet taste [1]. Furthermore, steviol glycosides derived from the *Stevia* plant are also determined as an extremely helpful part to prevent diabetes, obesity on the grounds that they help to increase insulin secretion and insulin sensitivity [3, 4]. Therefore, the *Stevia* plant can be used as a glucose blood regulator and weight control for obesity [1]. Preclinical studies referred that steviol glycosides originated from the *Stevia* plant help to prevent inflammation, hypertension, atherosclerosis**,** infection, dental caries, and some other fatal sicknesses like cancer, especially skin tumours. They also improve vasodilation, reduce blood pressure, and antioxidants [3, 4]. Besides, these natural sweeteners were proved to be stable in food and pharmaceutical technologies under different temperature values and pH conditions. They do not affect the flavour and taste of products [2]. Extensive consumption of the *Stevia* leaves has not caused any detrimental impacts on healthy human beings [4]. Steviol steviosides have been approved as a food additive (E960) with an acceptable daily intake (ADI) of 4 mg/kg body weight [1]. Steviosides group derived from *Stevia* plant also is natural sweetener applied as a sugar replacement in various countries such as United States, Canada, Brazil, South Africa and in the European Union [1, 5].

In recent years, steviosides group from *Stevia* plant was extracted with a variety of different methods, not only traditional techniques [6], but also non-conventional technologies such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pulsed electric fields, etc. In addition, several different kinds of solvents were used in extraction of total steviosides such as methanol [2], water, ethanol [7], glycerol [8], etc. Among which, aqueous and ethanol have been the two most popular solvents according to published studies. However, ethanol usage as a green solvent has been the preferable choice in comparison with water as it consists of the hydroxyl group which can lead to high extraction yields of natural compounds [9]. When it comes to the fundamental disadvantages of conventional extractions, there are various requirements such as the longer extraction time, the high temperature, the large amounts of the sample, a high energy requirement, the overall high cost, a poor extraction yield, and the high consumption of organic solvents which is often harmful on the environment. However, these negative influences can be decreased significantly by the application of modern extraction techniques [9, 10].

The technological processes are responsible for the level of degradation of biochemical compounds in the light of their sensitivity to light, thermolability, the damage of plant tissues, etc. [9, 11]. Therefore, the yield of biochemical compounds extracted from plants would primarily depend on proper solvents, extraction time, extraction temperature, a ratio of the sample, agitation, chemical and physical properties of the sample [9, 12].

For the abovementioned justifications, the main aim of this study was to analyse the chemical components in dried *S. rebaudiana* leaves and determine the influence of single factors on total steviosides content obtained including solvent/material ratio, temperature, and time extraction by ultrasound-assisted extraction with aqueous ethanol solution as the solvent.

MATERIALS AND METHODS

Sample preparation

The dried *Stevia* leaves (*S. rebaudiana* Bertoni) originate from Lam Dong province (Vietnam) and were purchased from Tan Phat HCM Co. Ltd., Vietnam. The moisture content of the sample was lower than

5%. The dried samples were ground and sieved for uniformity in size. After that, samples which passed through a sieve of 4 mm were packed in a polyethylene bag and stored at 4°C. These samples were used for all subsequent e xperiments.

Chemicals and reagents

Anthrone was purchased from HiMedia Laboratories (India) and stevioside (purity: ≥95.0%) was provided by Sigma Chemical Co. (Germany). All other chemicals and solvents were of analytical reagent grade.

Ultrasound-assisted extraction of stevioside

The dried *Stevia* leaves (2 g) were extracted with ethanol (70%, *v/v*) as a solvent by UAE in an ultrasonic bath (Elmasonic S60 H, 550 W, Germany). The experiments were implemented at various solid/liquid (SL) ratios (1/25, 1/50, 1/75, 1/100, 1/125, 1/150, and 1/175, g/ml); the extraction temperature ultrasound from 60 to 85°C, and at room temperature (30°C). Duration of the extraction process was 10 to 60 min. After the complete process, the extracts obtained were cooled to room temperature and vacuum-filtered with Whatman filter paper (No. 1) to remove the residue. After that, these specimens were determined in regard to the yield of total steviosides content (TSC) in the extract.

Determination of some components of dried *Stevia* leaves

Moisture, ash, crude fibre, total lipid, and total sugar content were determined according to AOAC 934.01; AOAC 923.03; AOAC 962.09; AOAC 920.39, and AOAC 968.28, respectively. Crude protein content was conducted using the Kjeldahl procedure (AOAC 978.04) [13]. Reducing sugar content was calculated by 3,5-dinitrosalicylic acid (DNS) assay and described by Miller [14]. This compound (DNS) reacted with reducing sugar to form 3-amino-5-nitrosalicylic acid, which strongly absorbed light at 540 nm. In addition, total polyphenols content (TPC) was estimated according to Folin-Ciocalteu (FC) method [15]. The reaction product, a result of the transfer of electrons in an alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, was determined spectroscopically at 738 nm. Besides, the pectin in dried leaves was extracted by microwave with oxalic acid as solvent and the amount of pectin obtained was estimated according to the study of Quoc [16].

Determination of total steviosides content (TSC)

The amount of total steviosides content in dried leaves was analysed by anthrone-sulphuric acid method as described by Yen and Quoc [6]. Firstly, dissolving 0.2 g of anthrone in 100 ml H_2SO_4 to prepare anthrone reagent which should prepare just whenever determined. Then, 6 ml of anthrone reagent and 2 ml of extract sample were mixed in a tube and shaken vigorously in an ice-bath to avoid high temperatures causing the solvent to evaporate. After that, the tube was heated to boil for 10 min. and cooled again. The mixture obtained was incubated for 30 min. at room temperature. The absorbance of the green solution sample received was measured at 550 nm. In addition, the stevioside standard was also prepared and built the standard curve.

Data analysis

All experiments were carried out in triplicates and the results were expressed in the form of a mean ± standard deviation (SD). Significant differences between the results were calculated by one-way analysis of variances (ANOVA) ($p<0.05$). Fisher's least significant difference (LSD) procedure was applied to compare the mean values. All statistical analyses were performed using the software Statgraphics Centurion (version 15.1.02, StatPoint Technologies, Inc., USA).

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS AND DISCUSSIONS

Chemical compositions of dried *S. rebaudiana* leaves

Table 1 shows the results obtained by the biochemical analysis of the dried *Stevia* leaves. In this study, the moisture of the sample was 2.53%, lower than some previous reports, whereas protein (10.67%) and lipid content (4.16%) were similar to the results of Gasmalla *et al.* [17] and Khiraoui *et al.* [18], who analysed biochemical compositions of the *Stevia*

leaves from different geographical regions. The low moisture of specimens in the present study could help to preserve the samples over long-time storage. The crude fibre accounted for 14.67%, which was in accordance with those recorded by Goyal *et al.* [19] and Serio [20]. Therefore, this material could be considered as a potential fibre supply to be applied in food technology. High ash content (7.26%) also pointed out that the *Stevia* leaves are a good source of minerals.

Table 1.

Chemical composition of dried *S. rebaudiana* leaves (g/100 g DW)

Components	Present study	[17]	[18]
Moisture	2.53±0.20	4.45–10.73	4.97–8.31
Total protein	10.67±1.23	12.44–13.68	11.75–16.23
Crude fibre	14.67±1.76	4.35–5.26	17.43–19.13
Ash	7.26±0.81	4.65–12.06	7.37–11.28
Total sugar content	18.26±1.96	ND	ND
Reducing sugar	7.20±0.87	ND	3.3–5.87
Total lipid	4.16±0.46	4.18–6.13	3.86–5.78
Pectin	0.010±0.005	ND	ND
TPC (mg GAE/g DW)	3.03±0.18	ND	ND

ND: not determined

Carbohydrates are the principal sources of energy for humans, especially sugar. Both reducing sugar (7.2%) and non-reducing sugar were found in the *Stevia* leaves with high values, higher than those analys\ed by Khiraoui *et al.* [18], which revealed a potential application of the extracts from *Stevia* leaves as a sweetener supplement. Except for some major compositions, this material also had the presence of bioactive compounds, for instance pectin (0.01%) and polyphenols (3.03 mg GAE/g DW). However, these compounds accounted for an insignificant level in the initial raw material. Besides, the extraction of bioactive compounds in samples would become more easily at a low pectin content because of low extract viscosity. In general, the different compositions of studies might be due to the different sources of samples or various horticultural techniques.

Effect of SL ratio on TSC in dried leaves

Figure 1 illustrates the influence of SL ratio on the steviosides extraction yield while the other extraction factors (ethanol concentration of 70% (*v/v*), extraction time of 30 min., and extraction temperature of 70°C) were remained unchanged.

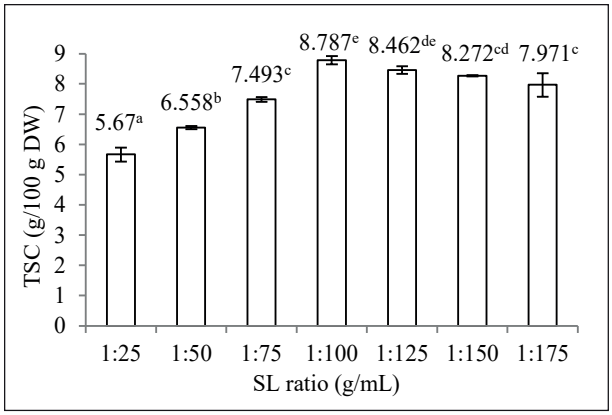

Figure 1.

TSC in extracts at various SL ratios

The yield of steviosides content obtained increased from 5.67 to 8.787 g/100 g DW of dried leaves when these leaves to extractant ratio increased from 1:25 to 1:100 (g/ml), respectively. Then, the amount of TSC reduced steadily at SL ratios between 1:100 and 1:175 (g/ml). The results recorded indicated that SL ratio strongly influenced on the extraction yield. The maximum TSC peaked at 8.787 g/100 g DW at the SL ratio of 1:100 (g/ml) and it was higher than that studied by Yen *and* Quoc [6] (8.36%), who also extracted total steviosides from *S. rebaudiana* Bertoni leaves by conventional extraction technique; and lower than that reported by Rao *et al.* [21] (10.9%), the authors also isolated total steviosides from this material by high pressure liquid extraction. Compared to another extraction method, the amount of solvent used in this study was lower than that in the study of Yilmaz *et al.* [22], who used the optimum SL ratio of 1:118 (g/ml) to isolate total steviosides by maceration method. Basically, solvent (ethanol 70%, *v/v*) can accelerate the mass transfer process from material to solvent by increasing the permeability of the plant tissues [23]. Additionally, in some cases, the solvent can break the bond between solutes and plant matrix [6]. If the amount of solvent to raw material is too small, total steviosides content in an initial material cannot be completely extracted up. On the contrary, if the amount of solvent to raw material is too big, this will cause a high process cost.

From the obtained data, it could be concluded that an appropriate SL ratio of 1:100 (g/ml) was of a

great importance in the achievement of high yields and cost-saving.

Effect of extraction temperature on TSC in dried leaves

In order to determine the most appropriate extraction temperature to recover TSC, extraction temperatures ranging from 30 to 85°C were investigated, while the remaining factors were unchanged (the SL ratio of 1:100 g/ml), extraction time of 30 min., and ethanol concentration of 70% (*v/v*)). The results in figure 2 showed the significant differences in TSC values at various temperatures. An increase in extraction temperatures would lead to an increase in the yield of TSC. At 75°C, the best amount of TSC obtained was 8.745 g/100 g DW; then, by increasing extraction temperatures from 75 to 85°C, the extraction yield of TSC slightly dropped. The best extraction temperature (75°C) in this study was quite higher than that reported by Yilmaz *et al.* [22], with optimal temperature of only 50°C with UAE process, while it was lower than that studied by Yen and Quoc [6], who used the conventional extraction technique to extract total steviosides from *S. rebaudiana* Bertoni leaves at 85°C.

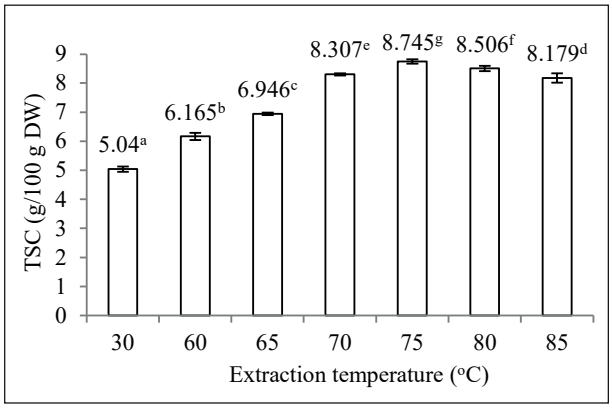

Figure 2.

TSC in extracts at various extraction temperatures

In this study, extraction temperature played an extremely important role during the extraction process as it helped enhance the yields of TSC obtained. At the optimal temperature, the plant tissues can be softened, leading to rising in the rate of solvent penetration into the material [24]. In addition, it also reduces the viscosity of solvents, goes up the solubility of phytochemical compounds in samples and disrupts certain chemical bonds to release compounds from the raw materials. Besides, the appropriate temperature promotes the diffusion coefficient and mass transfer rate [25-28]. However, in some previous reports, the high temperature could degrade some sensitive compounds for a longer time [25].

Hence, the extraction temperature of 75°C was considered to be the best temperature for the steviosides extraction process by ultrasound in this study.

Effect of extraction time on TSC in dried leaves

The amount of TSC in samples was extracted in various extraction times (from 10 to 60 min.) in order to select a proper time to improve the extraction yield, while ethanol concentration, SL ratio, and temperature were kept the same at 70% (*v/v*), 1:100 (g/ml) and 75°C, respectively. The yield of TSC increased rapidly from 7.354 to 8.894 g/100 g DW when the extraction time increased from 10 to 30 min., respectively. Then, from 30 to 60 min. of extraction time, there was a lightly downward tendency for TSC when extraction time increased (fig. 3). The UAE mechanism can be mainly explained according to the physical phenomena in which the cavitation produced in the solvent by the passage of ultrasonic irradiation results in disrupting the cell wall. So, the solvent can penetrate into the inner area and promote the diffusion of dissolved substances through the cell walls [29]. These phenomena are strongly influenced by ultrasonic time, temperature, and solvent. The long extraction time can facilitate the transition of the desired compounds into the solvent and enhance the extraction yield. However, a longer extraction time may induce the total steviosides extracted to oxidize and decompose during the extraction process at high temperatures. These results implied that UAE helped to decrease the optimum operation time significantly compared to the conventional method, for instance, Yen and Quoc [6] took 120 min. to isolate total steviosides extracted with distilled water as a solvent. Moreover, the present results were similar to those recorded by Liu *et al.* [29], who also extracted total steviosides from this plant by UAE for 32 min. However, extraction time in this study was shorter than that of Yilmaz *et al.* [22], who applied the MAE method to extract total steviosides for 16 min. The differences in results might be due to the difference in chemical compositions of materials, extraction methods, and solvents. Through the above analysis, 30 min. was chosen as the optimal extraction time for the UAE process.

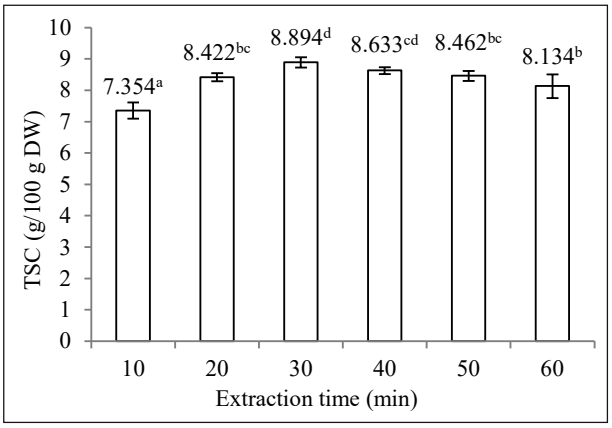

Figure 3.

TSC in extracts at various extraction times

CONCLUSIONS

The *Stevia* leaves, containing the high TSC, were considered to be an appropriate material for sweetener extraction. During UAE process, the optimal extraction conditions were ethanol concentration 70% (*v/v*), SL ratio of 1:100 (g/ml), an extraction temperature of 75°C, and an extraction time of 30 min. The novel technologies, the UAE method could be applied as a friendly environmental extraction method because of the decrease in ultrasonic temperature and time; use of ethanol as a solvent. These results obtained would be useful for some technical fields such as pharmacology, biotechnology, food, and chemistry.

Conflict of interest: Authors declare no conflict of interest.

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