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Pressurized Liquid Extraction of Steviol Glycosides from *Stevia rebaudiana* Leaves

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The present study aimed to evaluate the efficiency of pressurized liquid extraction (PLE) in obtaining steviol glycosides, natural sweeteners, from *Stevia rebaudiana* leaves. The extractions were conducted in an experimental apparatus operated in semicontinuous mode with fixed temperature, pressure and time at 120 ºC, 100 bar and 60 min, respectively, evaluating the effects of solvent to sample ratio (30 to 90 mL/g), concentration of ethanol in the extractor solvent (100 and 70 % v/v) for leaves with and without pretreatment. The results obtained were compared with the ultrasound-assisted extraction (UAE) conducted at 50 ºC, 60 min and solvent to sample ratio of 10 mL/g. Under pressurized conditions, the results showed greater extraction of sweeteners when pretreated leaves were used, reaching a yield of 70.94 ± 0.83%, which was higher than that obtained with the leaf without pretreatment (59.85 ± 1.14%). Increasing the solvent to sample ratio from 30 mL/g to 90 mL/g did not promote greater extraction in sweeteners from pretreated leaves (75.05 ± 1.89%), demonstrating that an excess of solvent in the medium does not result in higher yields. In parallel, little solvent (10 mL/g) did not contribute to obtaining sweeteners via UAE, promoting a yield of 46.78 ± 0.31%. The contents of sweeteners and mass obtained by PLE were about ~ 65% and ~ 55 % higher when compared to those obtained from UAE, respectively. The best results for sweeteners extraction (76.97 ± 2.70%) were obtained using pretreated leaves, at 30 mL/g and with 70% ethanol.

1. Introduction

The growing consumer awareness of the risks of diseases such as obesity and diabetes, has driven the industry in the search for low-calorie sweeteners, which aim to replace sugar. The most commercialized sweeteners today are synthetic compounds, such as saccharin and aspartame, which demonstrate long-term health impacts (Alkafafy et al., 2015). Therefore, aiming at the production of healthy foods, the search for natural sweeteners has been proposed and, in this sense, *Stevia rebaudiana* stands out. Cultivated for centuries in South America due to its sweetening properties (Puri et al., 2011), stevia has more than 20 steviol glycosides identified (Molina-Calle et al., 2017; Formigoni et al., 2018), among which are stevioside (Stv) and rebaudioside A (Reb A), the more abundant constituents and ~300 times sweeter than sucrose. Reb A has a better sensory profile than Stv, which is responsible for the characteristic bitter taste of the plant. In addition to their sweetening properties, these sweeteners do not cause caloric accumulation (Singh et al., 2019) and studies show that steviol glycosides have therapeutic properties, including antihyperglycemic, antihypertensive, anti-inflammatory, antitumor, anti-diarrheal, diuretic and immunomodulator effect (Ritu and Nandini, 2016; Ruiz-Ruiz et al., 2017).

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The main obstacle to the use of natural sweeteners in the food industry is the cost associated with their extraction and subsequent purification. The methodologies that have been proposed for the extraction of steviol glycosides are generally based on the use of superheated aqueous or alcoholic solvents, followed by several purification steps (Gasmalla et al., 2017; Ameer et al., 2017). Pressurized liquid extraction (PLE) is a technique that emerges as an alternative to conventional ones and it has as advantage the use of less solvents and energy, the greater selectivity and the better repeatability compared to other methods (Zhang et al., 2018), obtaining products with high purity and possibility of reducing the later stages. This process occurs with solvents at temperatures above their boiling point and below their critical point, associated with pressures sufficiently capable of maintaining them in the liquid state, allowing for increased solubility and promoting improvement in the mass transfer properties of the compounds of interest (Plaza and Turner, 2015). Due to these operating conditions, the physical-chemical properties of the solvents are modified, decreasing their surface tension and viscosity, causing an increase in the matrix's analyte diffusivity and desorption, due to the reduction of intermolecular interactions between analyte and matrix (Alvarez-Rivera et al., 2020). The pressure forces the solvent to penetrate areas of difficult access in atmospheric conditions, facilitating the extraction of analytes that are trapped in the pores of the matrix (Mustafa and Turner, 2011). Associated with the appropriate solvent, this type of extraction can promote appreciable yields in shorter periods of time (Pawliszyn, 2019), without causing losses in the composition of the obtained extract. Additionally, the pretreatment of the vegetable matrix can help to reduce the residual bitter taste of Stv (Formigoni et al., 2018), increasing the efficiency in obtaining sweeteners.
In this context, the aim of this work was to investigate the extraction by pressurized liquid (PLE) of steviol glycosides from *Stevia rebaudiana* leaves. The influence of the pretreatment, the effect of the ethanol concentration in the extracting solvent and the proportion of solvent and sample were evaluated, and the conditions that maximize the glycoside yield, mass yield and sweetener recovery were determined. The composition of the obtained extract was determined in relation to the total glycosides and the condition that led to the maximum yield in the PLE was compared to the result obtained by the ultrasound-assisted extraction (UAE).

2. Material and Methods

2.1 Sample and Reagents

Stevia rebaudiana Bertoni (Stevia UEM-13) plants were grown at the Nucleus of Research in Natural Products (NEPRON) located at the State University of Maringá (UEM, Paraná, Brazil). Harvested in the flower bud formation stage (~ 50 to 60 days after pruning), the leaves were separated from the stems, dried in an oven with air circulation at 60 °C for 8 hours, milled in a knife mill and had a final moisture content of <10% and average diameter of 0.30 - 0.60 mm. Part of the leaves were subjected to the ethanolic pretreatment (Formigoni et al., 2018), and later they were dried with the rest of the leaves and stored for the extraction. The composition in terms of sweeteners of the leaves used in this work with and without pretreatment are shown in Table 1. Ethanol (Honeywell, 99.9 % pure), ethanol (Anhydrol, 95.0 % pure) and distilled water were used as solvents in PLE and UAE. The deionized water (18 MΩ·cm) used in the chromatographic analysis was obtained by the Milli-Q plus system (Induslab, Brazil). All the reference standards were provided by Sigma-Aldrich (Brazil).

Table 1: Sweetener content in Stevia rebaudiana leaves before and after ethanolic pretreatment.

Analysis	Without pretreatment (g/100 g)	With pretreatment (g/100 g)
Stevioside	4.34 ± 0.04	4.08 ± 0.07
Rebaudioside C	1.92 ± 0.02	1.75 ± 0.01
Rebaudioside A	6.98 ± 0.04	6.83 ± 0.02
Total Glycoside	13.18 ± 0.06	12.76 ± 0.07

2.2 Pressurized liquid extraction

The experiments were carried out in a semicontinuous mode, as shown in Figure 1, and described by Rodrigues et al. (2017). The experimental apparatus consisted of a reservoir (SR) containing the solvent, which was continuously pumped by a high-pressure liquid pump (P). To enter the extraction bed (E) at the test temperature, the solvent passed through the preheating zone (P), which was monitored by a thermocouple (T).

At each end of the E there were synthesized steel filters. The oven (O) was heated to the desired temperature and, after reaching it, the E was allocated inside it with 2 g of stevia leaves, interspersed every 1 g with glass pearls.

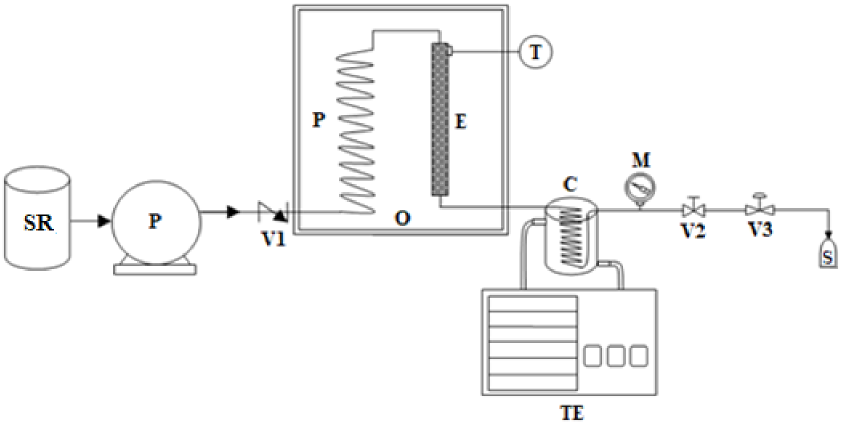

Figure 1: Semicontinuous experimental apparatus: (SR) solvent reservoir, (P) HPLC pump, (V1) check-valve, (O) oven, (P) preheater, (E) extractor, (T) thermocouple, (C) cooling unit, (TE) thermostatic bath, (M) manometer, (V2) pressure control valve, (V3) pressure reduction valve, and (S) sampling.

The extraction procedure is based on the heating of the system (O - Sanchis, BTT1050-00, Porto Alegre, Brazil) and the filling with solvent until the test pressure. Once the system was pressurized and the desired temperature was reached, the extractor was placed in the oven for 30 minutes in static time, after which the dynamic extraction was started. The samples (S) were collected after passing through a refrigeration system (C), connected to a thermostatic bath (TE). The system's pressure was monitored via a pressure indicator and controlled with a needle valve and a pressure reducing valve.

In the experiments, the extraction of steviol glycosides in leaves with and without ethanolic pretreatment was investigated. The proportion of ethanol in the extracting solvent of 100 and 70 % (v/v) and the proportion of solvent in the sample (R-L:S) of 30 and 90 mL/g were evaluated. The temperature and pressure used for the extractions were 125 C and 100 bar, respectively, during the fixed time of 60 min. After the extraction period, the solvent was removed in a rotary vacuum evaporator (Marconi, MA 120) and dry extract was stored in a desiccator. The mass yield was calculated according to Eq (1), where q_o is the extract mass obtained (g) and q_s is the leaf mass (g) used in the experiment.

$$yield\ (\%) = \left(\frac{q_o}{q_s}\right) \times 100 \qquad (1)$$

2.3 Ultrasound-assisted extraction

An ultrasonic bath with indirect contact and heating control (Ultronique, Q 3.0/40 A, Eco-Sonics), with a frequency of 40 kHz and power of 110 W, was used for the ultrasonic extraction. A flask (250 mL) containing the milled leaves and the solvent was connected to a condenser coupled to a cooling bath (Marconi, MA 184), which were positioned in the center of the ultrasonic bath. To evaluate the influence of the ultrasound on the extraction of steviol glycosides, 10 mL/g of 70% ethanol solvent were sonicated for 60 min, at 50 °C, with pretreated leaves (13.03 ± 0.02 g/100g of glycosides). After the extraction period, the leaves were separated by filtration, the solvent removed and the mass yield calculated according to Eq (1).

2.4 Quantification of steviol glycosides by HPLC

The total glycosides present in the leaves after the extraction steps were identified and quantified using a High Performance Liquid Chromatograph (HPLC). After extraction, the samples were concentrated to dryness in a vacuum rotary evaporator (Marconi, MA 120), being subsequently redissolved with 10 mL of the mobile phase (acetonitrile:deionized water, 80:20, v/v), as described by Dacome et al. (2005). For the analyses, a liquid chromatograph model CG 480-C (Brazil) equipped with a 5 µm (125 × 4.6 mm) NH_2 column was used, operated isocratically with a flow of 0.75 ml/min at room temperature and coupled to a Waters 410 DRI detector (coupled to an index refraction detector S:32).

2.5 Statistical analysis

All the analyses were performed in triplicate and the results were expressed as mean values ± SD. To verify the influence of the parameters evaluated in each step on the results obtained, analysis of variance (ANOVA; Excel® 2010 software) and the Tukey test, with a 95 % confidence interval, were carried out.

3. Results and Discussion

Table 2 shows the results of the extraction of steviol glycosides in terms of stevioside (Stv), rebaudioside C (Reb C) and rebaudioside A (Reb A), as well as the mass and sweetener yields obtained by PLE at temperature, pressure and time fixed of 125 □C, 100 bar and 60 min, respectively. Through the analysis of the data presented in Table 2, it is possible to verify that the ethanolic pretreatment had an influence on the mass yield and the content of sweeteners obtained by PLE, without causing significant losses in the composition of the extract obtained (experiments 1 and 2). The higher mass yield obtained (43.31%) resulted from an increase in the L:S ratio from 30 to 90 mL/g, however, significantly reduced the levels of Stv and Reb A in the extract (experiments 3 and 4), indicating that both are similarly affected by this factor, which is expected by the chemical similarity between these two glycosides. Increases in glycoside yields, proportional to the increase in the L:S ratio, were previously reported, indicating the influence of this variable in the extraction process (Martins et al., 2017; López-Carbón et al., 2019). The proportion of ethanol (EtOH) in the extractor solvent affected the glycosides yields, mass and sweeteners (experiments 2 and 3). Although the decrease in the proportion of ethanol from 100 % to 70 % has promoted a significant reduction in the contents of Stv, Reb C and Reb A, an increase in mass and sweetener yields has been verified. This is due to the change in polarity and extraction capacity that the water content in the ethanol promotes to the solvent (Celaya et al., 2016), making the dissolution of the constituents more effective (Carbonell-Capella et al., 2016), resulting in higher yields under lower proportions of EtOH (Martins et al., 2016; Medina-Medrano et al., 2019).

Table 2: Effect of variables on the yield of total glycosides, mass and sweeteners in the PLE of leaves with and without ethanolic pretreatment.

Run	Leaves	EtOH (%)	R-L:S (mL/g)*	Total Glycosides (g/100 g)**			Yield (%)	Sweeteners (%)**
				Stv	Reb C	Reb A		
1	Without pretreatment	100	30	12.51 ± 0.04^{a}	5.77 ± 0.38^{a}	13.48 ± 0.67^{a}	24.67	59.85 ± 1.14^{a}
2	With	100	30	11.28 ± 0.12^{bA}	5.88 ± 0.42^{aA}	13.42 ± 0.04^{aA}	29.21	70.94 ± 0.83^{bA}
3	pretreatment	70	30	9.41 ± 0.73^{B}	4.79 ± 0.21^{B}	12.71 ± 0.26^{B}	36.02	76.97 ± 2.70^{B}
4		70	90	7.86 ± 0.12^{C}	3.88 ± 0.54^{B}	10.07 ± 0.18^{C}	43.31	75.05 ± 1.89^{B}

*R-L:S, Ratio leaf:solvent; **Different lowercase letters in the same column indicate significantly different values between tests with leaves with and without pretreatment ($p < 0.05$); Different capital letters in the same column indicate significantly different values between tests with pretreated leaves ($p < 0.05$).

Studies report that pretreatments in vegetable matrices aims to improve the transfer of mass and heat, leading to shorter extraction times, less solvent consumption, energy savings, better yields, better quality and greater purity of compounds extracted (Amiri-Rigi et al., 2016; Llavata et al., 2020). Recently proposed, the ethanolic pretreatment in stevia leaves aims to improve the sensory profile of the obtained extract, reducing the sensory characteristic of bitterness caused by Stv (Formigoni et al., 2018). Regarding extraction, the PLE process has stood out in comparison to other emerging processes, such as microwave assisted extraction (MAE) (Ciulu et al., 2017), showing viability in the proposed investigation. For this purpose, in this work, the association of the pretreated vegetable matrix with PLE promoted appreciable mass (29.21%) and sweeteners yields (71.51 ± 0.84%), higher than those obtained through the leaf without pretreatment (24.67% and 63.22 ± 1.21%, respectively).

Table 3 presents the results of the best condition proposed in the PLE in comparison to the results obtained by the UAE, in relation to the extract composition, mass and sweeteners yields. Through Table 3, we can see that the PLE had yields higher than the UAE, corroborating with results found by Plaza et al. (2012), where PLE promoted yields of 36.43% while the UAE generated a yield of 4.79%, under the extraction of *Chlorella vulgaris*. Although no significant differences were obtained in relation to total glycosides when comparing the two extraction techniques, in which PLE promoted 26.91% and UAE resulted in 26.15%, PLE provided yields in sweeteners ~65% higher than those obtained by the UAE. This is mainly due to the fact that the process acts through the interaction between temperature and pressure, which, keeping the solvent in a liquid state and above the boiling point, promote better analyte solubility, faster diffusion, lower solvent viscosity while weakens the interactions between the sample solution and the matrix (Kovačević et al., 2018).

Table 3: Yield in total glycosides, mass and sweeteners in PLE and UAE.

Extraction method		PLE*	UAE*
Total Glycosides (g/100 g)	Stevioside	9.41±0.73^{a}	10.47±0.10^{a}
	Rebaudioside C	4.79±0.21^{a}	5.71±0.01^{b}
	Rebaudioside A	12.71±0.26^{a}	9.97±0.06^{b}
Yield (%)		36.02	23.24
Sweeteners (%)		76.97±2.70^{a}	46.78±0.31^{b}

PLE of pretreated leaves, R-L:S 30 mL/g, 70 % EtOH, 125 □C, 100 bar and 60 min; UAE of pretreated leaves, R-L:S 10 mL/g, 70 % etOH, 50 □C and 60 min; *Different letters on the same line indicate significantly different values ($p < 0.05$).

4. Conclusions

The pressurized liquid extraction of steviol glycosides from *Stevia rebaudiana* leaves was investigated. It can be concluded that the ethanolic pretreatment of the leaves improves the mass and sweetener yield, without causing loss of the extracted compounds. Reduction of the ethanol content in the extracting solvent from 100% to 70% promoted an increase in mass yields (36.02%) and in sweeteners (76.97%) in the obtained extract. The application of a higher solvent flow (90 mL/g) improves the extraction efficiency, providing greater mass yields (43.31%), however, significant loss of Stv and Reb A are observed. PLE promoted yields of ~56% and ~65% of mass and sweetener recovery higher than that obtained by the UAE, respectively.

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