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Sweeteners from Pre-Treated Stevia Leaves: Evaluation of The Effect of Extraction Cycles by Semi-Continuous Percolation

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Stevia rebaudiana could be used to makes to produce nutraceuticals and functional foods instead of sucrose and artificial sweeteners. Due to the nutritional and technological advantages of sucrose, innovative approaches for extracting sweeteners from Stevia leaves have been developed and optimized over the years. The purpose of this study was to evaluate the effect of extractingsteviol glycosides from leaves of a new variety of *Stevia rebaudiana* that had been pre-treated with ethanol. The extractions were carried out in a semi-contiunous mode in a percolator, evaluating the effects of the proportion of solvent to sample (ratio 1/10 and 1/40) and temperature (30 °C and 100 °C) for leaves pre-treated with ethanol. The Tukey Test ($p<0.05$) was utilized to identify sweeteners through gas chromatography.The highest temperature and proportion of solvent to sample were the best conditions to get an extract rich in sweeteners (13.93 g/100g of dry extract). Under these conditions, the application of two cycles proved to be sufficient to achieve a satisfactory recovery of glycosides in the extract (~93%), while allowing for a lower solvent consumption. Temperature reduction (30°C) decreases glycoside recovery (15.71%) and mass yield (11.14%) in the first extraction cycle. However, temperature ceased was no longer a significant factor ($p<0.05$) when the solvent volume was reduced to a 1/10 ratio. It is thought that particles with a smaller diameter could be used to gethigher levels of sweeteners, in future.
Keywords: *Stevia rebaudiana* Bertoni; cycles; conventional extraction; sweeteners.

1. Introduction

Consumers are increasingly looking for more natural and healthy food products, according to Castro-Muñoz et al. (2022). In this context, numerous studies have elucidated the numerous advantages that Stevia rebaudiana sweeteners offer for human health, highlighting its functional and nutraceutical attributes (Ahmad et al., 2020). This is attributed to the presence of numerous phytochemical compounds (Koubaa, et al., 2015) including antioxidant properties, antiglycant properties (Ali et al., 2022), and effects as antidiabetics agents (Zorzenon et al., 2019).

In addition to the significant quantity of antioxidant compounds (Ahmad et al., 2020), as well as significant macro- and micronutrients (Koubaa et al., 2015), stevia leaves possess a complex mixture of diterpene glycosides in their composition (Gasmalla et al., 2014). Commercial extracts are predominantly composed of stevioside (>80%) or rebaudioside A (>90%). Rebaudioside A (Reb-A) is more soluble and has a sensory profile closer to that of sucrose than Stevioside (Stv) which is responsible for the characteristic bitter taste of Stevia (Bursać Kovačević et al., 2018).

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Scientist have found out that stevioside doesn't cause mutations, tumors or cancer. Likewise,no allergic reactions have been observed when it is used as a sweetener (Yildz-Ozturk et al, 2014). In numerous nations, dried stevia leaves and their extracts of varying purity were initially approved as dietary supplements, necessitating marketing claims of health benefits. Until 1995, the Food and Drug Administration (FDA) in the United States prohibited the utilization of stevia and its extracts as a tabletop sweetener. In 2008 and 2009, a substance called rebaudioside A, wich is very pure, was given GRAS (generally recognized as safe) status (Wölwer-Rieck , 2012).

According to studies, pre-treatments in plant matrices aim to improve mass and heat transfer, leading to shorter extraction times, lower solvent consumption, energy savings, better yields, better quality and greater purity of the extracted compounds (Amiri-Rigi et al., 2016; Llavata et al., 2020). Recently proposed, ethanolic pre-treatment for Stevia leaves aims to improve the sensory profile by reducing the characteristic bitterness caused by stevioside. This will increase the efficiency in obtaining sweeteners (Formigoni et al., 2018).

The ethanolic pretreatment of stevia leaves has the capability to enhance the yield of dough and sweeteners without compromising the extraction compounds (Raspe et al., 2021). Ciotta et al. (2022) obtained extracts with a high recovery of steviol glycosides (93.80%) by employing the conventional extraction technique by semi-continuous percolation with boiling water. Analyses of the microstructure revealed that samples treated with ethanol exhibit structural changes that make the cells more compact with thinner walls, as well as a loss of intracellular air (Rojas and Augusto, 2018) that promotes better dissolution of the components (Wang et al, 2019).

Researchers around the world are working on developing extraction procedures that are cost-effective, green, and efficient without sacrificing the flavor of the extract (Yildiz-Ozturk et al, 2014). Evaluation of the extraction and purification of steviol glycosides obtained from stevia leaves, revealed a large body of literature involving both conventional and modern methods (Jentzer et al, 2015; Formigoni et al, 2021).

Modern industrial purification processes still use conventional methods or combine classical and modern methods due to the yield and cost of equipment. This still limits the large-scale use of an unconventional process by industries (Formigoni et al, 2021). Hence, it is evident that the tradicional extraction methodologies continue to play a significant role in numerous studies. A large proportion of undesirable compounds for commercial stevia extracts, but with bioactive potential that are discarded can be recovered in purification steps (Formigoni et al., 2019). This indicates that pretreatment combined with nanofiltration can be a good strategy for recovering these compounds.

Stevia UEM-13 is a Brazilian elite variety with a high level of rebaudioside A, the sweetener with the best sensory profile. In this particular context, the objective of this study was to investigate the extraction of steviol glycosides (SG) through semi-continuous percolation from pre-treated of a new variety of *Stevia rebaudiana* leaves. Cycles in the extraction step with water as solvent were investigated as a function of conditions that maximize glycoside recovery (G_R) and extraction yield (Y_E), with the composition of the obtained extract determined in relation to SG.

2. Materials and Methods

For the experiments, we used *Stevia rebaudiana* leaves from the Semina Stevia UEM-13 variety. They were grown at the Center for Research in Natural Products (NEPRON) at the State University of Maringá in Paraná, Brazil (23°24′ and 21°9′ S; 51°56′ and 22°0′W). The leaves were picked at the height of their growth (~50-60 days after being cut). The plants were dried in an oven with forced air circulation (60°C) for 8 hours until their moisture content was below 10%. The leaves were separated from the stems and ground in a stainless steel knife mill with a 2 mm sieve opening (Marconi, TE 340) after being separated from the stems. The crushed leaves were then treated with ethanol (Formigoni et al., 2018) to get a material with more sweeteners. The leaves were conditioned in a colum with absolute ethanol for 30 minutes, with subsequent continuous elution at a flow rate of 5 mL/min. The leaves were dried in a 60°C oven for 8 hours. The pretreated ground leaves were ategorized according to the stablished Tyler sieve series (Bertel, ASTM). The sweetener content of these fractions was quantified using HPLC according to the methodology described by Dacome et al. (2005). For analysis, 2 ml of the liquid extract were diluted in 8 ml of acetonitrile and the obtained solution was sonicated for 5 min (Ultronique, Q 3.0/40 A/110 W, Eco-Sonics), filtered 3 times (hydrophobic membrane, 0.5 µm, Millipore) and 20 µL were injected into the high-performance liquid chromatography system (Gilson, model 307), consisting of a low-pressure pump (Gilson, model 5. SC), refractive index detector (IR 133, Gilson), column oven (West, model 2300) and NH2 analytical column (125 mm x 4.6 mm x5 µm, Scientific Term, HyperSil Gold Amino). The composition of the leaves in terms of sweeteners was 13.6 ± 0.16 steviol glycosides. The glycosides were solely evaluated in the pre-treatment samples, as the same research group has demonstrated (Ciotta, et al., 2022) that ethanolic pre-treatment is highly selective and capable of removing classes of substances that leave the sweetner content in untreated leaves practically unchanged.

For sweetener extraction, crushed pre-treated stevia leaves (2 mm) were placed on analytical filter paper and dried (5g) were percolated with deionized water.
The conditions of temperature and leaf-solvent ratio are listed in Table 1. Since from the third extraction cycle onwards, there was no significant difference for the composition of Stevia sweeteners, this process was repeated two more times (Ciotta, et al., 2022).

Table 1: Experimental conditions evaluated in semi-continuous percolation extraction of compounds from pre-treated Stevia rebaudiana leaves.

Test	Temperature (°C)	Leaves/Sovent ratio
1	30	1/40
2	100	1/40
3	30	1/10
4	100	1/10

The experiments were usedt to test the temperature and renewal of the solvent through the application of 3 extraction cycles, against the response variables G_E, G_R and Y_E. At the conclusion of the three cycles, the filtrate was concentrated until the solvent was completely eliminated (using the Marconi vacuum rotary evaporator, MA 120). The extraction yield (Y_E) was calculated based on the initial mass and mass of the leaf sample extracted in each cycle. Dacome et al., (2005) determined the levels of glycosides in the extract (G_E), while the recovered glycosides (G_R) were calculated using to equation 1.

$$G_R = \left(\frac{G_E}{GS_L}\right) x100 \quad (1)$$

Where G_E denotes the mass of glycosides in the extract (g) and GS_L denotes the mass of initial glycosides in the extract leaf sample (g of leaf). To verify the influence of variables on the results obtained an analysis of variance (ANOVA; Statistica 8.0 software) and Tukey's test were performed, with a confidence interval of 95%.

3. Results and Discussion

Table 2 presents the results obtained from the investigated dataset (Table 1), in terms of GS, as well as in terms of the G_E, G_R and Y_e response variables.

Table 2. Content of steviol glycosides in the extract (G_E), recovery of sweeteners (G_R) and mass extraction yield (Y_E) of the semi-continuous percolation extraction assays of pre-treated Stevia rebaudiana leaves.

Test	Cycle	G_E (g/100g of dry extract)	G_R (%)	Y_E (%)
1	I	2.17 ± 0.02^{Bb}	15.71 ± 0.31^{Bb}	11.14
	II	3.42 ± 0.03^{Ba}	24.78 ± 0.39^{Ba}	20.27
	III	1.69 ± 0.02^{Bc}	12.23 ± 0.33^{Bc}	0.24
2	I	9.42 ± 0.13^{Aa}	66.23 ± 1.87^{Aa}	29.19
	II	3.68 ± 0.05^{Bb}	26.64 ± 0.79^{Bb}	7.90
	III	0.83 ± 0.01^{Cc}	6.02 ± 0.09^{Cc}	2.10
3	I	0.17 ± 0.01^{Cc}	1.24 ± 0.09^{Cc}	1.88
	II	1.92 ± 0.01^{Cb}	13.88 ± 0.77^{Cb}	15.60
	III	2.67 ± 0.05^{Aa}	19.36 ± 1.70^{Aa}	12.28
4	I	0.31 ± 0.00^{Cc}	2.23 ± 0.02^{Cc}	2.27
	II	4.39 ± 0.01^{Aa}	31.84 ± 0.13^{Aa}	17.22
	III	2.80 ± 0.02^{Ab}	20.29 ± 0.28^{Ab}	6.67

Different lowercase letters in the same column indicate significantly different values between runs for each assay ($p<0.05$). Different capital letters in the same column indicate significantly different values between runs for each cycle ($p<0.05$).

The data presented in Table 2 show that there was a significant difference ($p<0.05$) between cycles and tests. The most amount of material was extrated mass yield obtained (29.19%) and glycoside recovery (~66%) during the first extraction cycle in test 2, where the experimental conditions are maximum (Table 1).

Approximately 93% of the total glycosides were recovered with only two extraction cycles (Test 2), allowing for lower solvent consumption. Ciotta et al. (2022) found a similar result (93.8%) after using 5 extraction cycles. Other studies have also reported higher recoveries of stevia sweeteners using higher temperatures (Ciotta et al., 2022; Das et al., 2015).

The temperature is a significant parameter in the extraction process, as several aspects of a solvent such as diffusivity, viscosity, and solubility are affected by this parameter. Temperature affects on the extraction process by changing the diffusivity and solubility of the solute. The increase in temperature increases the diffusion of the solute, providing an improvement in extraction (Rouhani, 2019).

Increasing the temperature makes it easier to get soluble and insoluble impurities in the extract. It is still estimated that microfiltration can easily remove by insoluble solids, obtaining extracts with more than 80% purity (Ciotta et al., 2022). Another factor to consider is the increased in production costs due to the high amounts of solvent and energy required in high-temperature processes (Rouhani, 2019).

High temperatures can also affect the cavitation phenomenon (Milani et al., 2020), which affects yield by causing the degradation of compounds that affect the purity of the sweetener (Das et al., 2015).

It was also confirmed that the temperature reduction in test 1 decreased the recovery of glycosides in the first cycle (15.71%) compared to test 2 (66.23%). This is because reducing in temperature decreases the rate of diffusion and solubility of the analytes (Rouhani, 2019), promoting a more superficial penetration of the solvent through the cell wall (Alvarez-Rivera et al., 2020).

However, temperature no longer was a significant factor ($p<0.05$) when the solvent volume was reduced to a 1/10 ratio in the first extraction cycle (Tests 3 and 4). In this case, the concentration gradient established by the solvent is reduced, thereby hindering the diffusion rate. In cycles 2 and 3, glycoside recovery and extraction yield are more significant ($p<0.05$) because the solvent absorption capacity increases when it is renewed (Jentzer et al., 2015).

The use of larger volumes of solvent for extracting plant material has been previously reported, and the best results found here (1/40 ratio) are within the range (1/35 to 1/50) indicated by other authors for *Stevia rebaudiana* (Gasmalla et al., 2014; Koubaa, Roselló-Soto, Žlabur, et al., 2015).

The volume of solvent in the sample causes an increase in the contraction gradient and driving force and consequently increases the diffusion rate (Jentzer et al., 2015; Maran et al., 2017), making the extraction process more efficient. Also, the parts that are soluble components dissolve better in this situation.

This effect may also be related to the fact that Stevia leaves have a high wettability (Mishra et al., 2010), promoting rapid solvent absorption by the matrix and excessive swelling. Furthermore, the addition of extraction cycles allows for solvent renewal can disrupt the process and increase its absorption capacity (Jentzer et al., 2015). Another thing to remember is that most studies use water because it is green solvent. However, this solvent may be less selective because it is more soluble than the desired analyte (Castro-Puyana et al., 2017).

Extraction in plant matrices is a mass transfer process that depends mainly on how accessible of the compounds are to the solvent. Cell disruption strategies are used to improve yield and mass transfer (Romero-Díez et al., 2019; Ummat et al., 2021). One approach strategy is to grind the solid matrix into smaller particles. This mechanical process can give higher levels of sweeteners promoting greater optimization of extraction by semi-continuous percolation. As the particle diameter decreases, the contact area of the solid matrix with the solvent increases, wich favors the mass transfer of solutes through the diffusion process (Preece et al., 2017).

4. Conclusions

Evaluation of semi-continuous percolation extraction of steviol glycosides from pre-treated *Stevia rebaudiana* leaves was conducted. It can be concluded that the application of two cycles of GS extraction from Stevia leaves was enough toto get the maximum yield, allowing less solvent consumption and a cleaner extraction. The highest yields were obtained from the combination of higher temperature and solvent availability (100°C and 1/40), whose G_R and Y_E were ~98% and ~39%, respectively, with a G_E content of 13.93g/ 100g of dry extract. It is expected that it will be possible to obtain higher levels of sweeteners using particles with reduced diameter in future works.

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