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Effect of enzymatic pretreatment on the extraction yield of *Stevia rebaudiana* leaves

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Abstract

Stevia rebaudiana (Bert.) is a plant, which has in its constitution, especially in the leaves, steviol glycosides with sweetener power. This plant is worldwide studied with the main aim of effectively extract these compounds. The aim of this study was to evaluate the effect of an enzymatic pretreatment on the S. rebaudiana leaves by using cellulase enzyme on the yield of an extraction using conventional methods. The pretreatment was made in different durations (15, 30, 45, 60 min) with a relation of 1:5 (w/v), at 50°C and 150 rpm, followed by an aqueous extraction at 1:10 (w/v), 50°C and 120 rpm. It was verified that the pretreatment duration directly influences the yield of glycosides extraction, with 1 hour being defined as the best duration. The enzymatic pretreatment before the extraction demonstrated to be very effective for the global extraction yield, since there was a raise of 34.4% in the total yield when compared with the same extraction without pretreatment. A high extraction yield (71%) of glycosides originally present in the leaves was also observed in treated leaves extract when compared with untreated leaves extract (56%) in just one cycle. Among them, the one which exhibited higher content was rebaudioside A, which has superior sensory characteristics. It is concluded then that the enzymatic pretreatment before sweeteners extraction of *S. rebaudiana* leaves is a viable alternative, with similar recovery to modern techniques. However, it is presented as a simple, fast, inexpensive and advantageous methodology in relation with other techniques which are expensive and may present application difficulties in industrial scale.

Introduction

Stevia rebaudiana (Bert.) is a perennial shrub which belongs to the Asteraceae family, native from the Amambay region, in the northeast region of Paraguay. It also occurs in neighboring regions, as Brazil and Argentina. It features, especially on its leaves, high content of diterpene glycosides which have sweetening characteristics (about 4-20% on dry leaf). Among these compounds, stevioside (4-20%), rebaudioside A (1-3%) and other steviol glycosides in minor proportions (<0.1%) stand out. Stevioside and rebaudioside A have more sweetness potential than sucrose (280 and 450 times, respectively) and show good biocompatibility (Soejarto, 2002; Ghanta *et al*., 2007; Prakash *et al*., 2008).

Although usage of Stevia sweetner had already been widespread for decades in American and Asian countries (Kroyer, 2010), in November 2011 the European Food Safety Authority – EFSA, granted the GRAS (Generally Recognized as Safe) for the purified steviol glycosides, officializing the power use of this sweetener as a food additive/sweetener (EFSA, 2011), and because of its heat stability, it presented utilization potential in a wide range of industrial applications in food, such as beverages in general, desserts, sauces, bakery products, dairy products, among many others (Lemus-Mondaca *et al*., 2012).

In creole varieties, stevioside is the major constituent in Stevia leaves and beyond the sweetness, it has an undesirable bitter flavor when compared with rebaudioside A (rebA), which partially restricts its use for human consumption (Ye *et al*., 2013). Therefore, the search for new genetically modified varieties in order to obtain plants with greater rebA content becomes the main aim of research groups and industries, worried with the improvement and use of this natural sweetener source (Dacome *et al*., 2005).

Studies have evaluated different methods to extract the steviol glycosides using different techniques, from conventional alternatives such as hot water extraction or Soxhlet extraction (Pól *et al*., 2007; Shukla *et al*., 2009; Kim *et al*., 2011) or even complex techniques such as supercritical CO_2, ultrasonical or microwave-assisted (Liu *et al*., 2010;

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Yildiz-Ozturk *et al.*, 2014; Periche *et al*., 2015). Among the various extraction methods, hot water process is a viable method both operationally and economically and safer from a health point of view when compared with extractions performed using chemical solvents (Ray and Majumdar, 2012).

In the search for a higher extraction yield, modern techniques have been gaining notability. A studied methodology consists of the degradation of the plant's cell wall that makes compounds of interest, mainly glycosides, more accessible for extraction. In ultrasonically extraction technique, the procedure induces the solvent penetration inside the cell-matrix, which leads to a change in structure and, consequently, an improvement in the mass transfer (Toma *et al*., 2001). Another studied method is the microwave technique, in which the friction resulting from molecular movement contributes to the rapid heating of vegetal matrix, increasing yield and decreasing time compared with conventional methods (Whang *et al.*, 2008). In industry, such tissue desnaturation is most often achieved by thermal processes (e.g., using steam or hot water), which consume large amounts of energy. In order to reduce costs and create more viable alternatives, chemical or enzymatic treatments have also been used (Puri *et al.*, 2012; Barba *et al.,* 2014). Furthermore, studies show that a Stevia leaves pretreatment may facilitate the extraction process and improve the quality of the sweetener obtained, contributing to the reduction of bitter aftertaste and process costs (Pasquel, 2000).

In this context, the aim of this study was to evaluate the effect of an enzymatic pretreatment on the extraction yield of S. rebaudiana leaves.

Materials and Methods

Plant selection

S. rebaudiana (Bert.) plants from the seminal variety (UEM-13) were obtained in the experimental site at the pilot unity of the Center of Studies in Natural Products – NEPRON, at the State University of Maringá. Its leaves and stems were collected during the stage of maximum vegetative growth and previously dried in forced air circulation oven at 60°C until reaching a humidity lower than 10%. After this procedure, leaves were separated from stems and branches and then previously milled for use in subsequent steps.

Materials

All chemical solvents and analytical grade reagents used in this study were acquired from Induslab Co (Londrina, state of Paraná, Brazil). Stevioside and rebaudioside-A patterns were obtained at NEPRON. To perform the enzymatic pretreatment it was used Celluclast 1.5 enzyme, derived from Trichoderma reesei (Novozymes A/S, Bagsvaerd, Denmark) courtesy of Latin American LNF.

Determination of steviol glycosides content in stevia leaves

The extraction of steviol glycosides from Stevia leaves was performed according to Dacome *et al.* (2005). Leaves (2.0 g) were extracted from a seminal variety Stevia UEM-13 milled in approximately 70mL of water at 100°C during 5 minutes and constant stirring, followed by vacuum filtration. This extraction procedure was repeated two more times (three cycles) and the final extract for the volume of 250 mL was completed. Then, 10 ml of the solution was dried in rotary evaporator and analyzed by HPLC.

Enzymatic pretreatment

The enzymatic treatment was carried out according to Puri *et al*. (2012), with some adjustments. Dry leaves of *S. rebaudiana* were placed in erlenmeyer with 0.1 M sodium phosphate buffer with pH of 4.7 and 2% of cellulase enzyme (w/v) (1: 5). The contents were stirred on shaker 150 rpm at 50°C for 15, 30, 45 and 60 minutes to be aware of the influence of time on the overall yield and content of the extracted glycosides. Given the time, pretreated leaves followed to the extraction step.

Extraction of steviol glycosides

Extraction of steviol glycosides occurred by conventional methods using water as solvent. Pretreated leaves were placed in deionized water (1:10) and incubated at 50°C for 3h under constant stirring (120 rpm). Then, the content was filtered and centrifuged at 3600 rpm for 3 minutes in order to remove suspended particles. The centrifuged extract was dried in rotary evaporator and subsequently analyzed in HPLC. It was also made extraction of leaves without enzymatic treatment under the same conditions for posterior comparison.

Determination of glycosides by high-performance liquid chromatography (HPLC)

The content of total glycosides including stevioside, rebaudioside A and C in aqueous extracts were determined by high-performance liquid chromatography in isocratic sistem liquid chromatograph with column NH_2 of 5 μm and dimension of 125x4.6 mm coupled with refractive index detector S:32, using acetonitrile and water as

mobile phase (80: 20 v/v) with flow 0.5 mL/min.

Statistical analysis

All analyzes were performed in triplicate. The results are presented as average values with standard error and were analyzed statistically using one-way ANOVA and Tukey test. Statistical significance was accepted at a level of $p < 0.05$ using statistical program STATISTICA 8.0.

Results and Discussion

Characterization of glycosides present in the leaves of the variety S. rebaudiana

The seminal variety Stevia UEM-13 showed total glycosides content in leaves of 10.5%. When compared with other studies (Table 1), the present study obtained higher values for total glycosides. Thus, due to the high content of glycosides present in the leaf, and among them rebaudioside A in greater quantity, it can be claimed that the variety used is considered elite when related to wild varieties.

Effect of pretreatment duration

The Table 2 expresses the extraction yield of glycosides during different durations of pretreatment. It can be observed that rebaudioside A was the predominant steviol glycoside in the enzymatic aqueous extract, followed by rebaudioside C and, finally, stevioside. At times 30 and 45 min, there was a decrease in glycosides extraction, and at time of 60 min, the extraction yield glycosides was the highest.

Influence of enzymatic pretreatment on extraction yield

An increase of 34.4% in global extraction yield (crude extract in mg/g) was observed in extracts of pretreated leaves with cellulase enzyme (AEPL) when compared with the same traditional extraction method in untreated leaves (AEUL) (Table 3). Yildiz-Ozturk *et al.* (2014), obtained a maximum extraction yield of 489 mg/g in a subcritical water extraction at a rate of 11 mg/g of dry leaves per minute.

It is noted the total glycoside content obtained in AEPL was higher than in AEUL, occurring with pretreatment leaves an increase of 15% in extraction of glycosides, which features a better quality of extract obtained with treatment. Also, 71.39% of total glycosides from treated leaves were extracted in only one extraction cycle, which can be considered high efficiency. This increase, both yield mass crude extract and of the total of glycosides, can be explained by the fact that cellulose enzyme has good degradation capacity of cell wall polysaccharides, such as xyloglucans and heteroxylans, leading to a cell wall disintegration, facilitating the solvent access to the compound of interest (Puri *et al.*, 2012).

Table 1. Content of total glycosides obtained from stevia uem-13 leaves compared with other works cited in the literature.

		References		
Glycosides	Stevia UEM-13	Kovylyaeva *et al.* (2007)	Gardana *et al.* (2010)	Atteh *et al.* (2011)
Stevioside	4.06	5-6	5.8	6.5
Rebaudioside C	2.03	0.3-1.3	Nr	1.3
Rebaudioside A	4.41	0.3-1.3	1.8	2.3

Nr: not reported

Table 2. Extraction yield of steviol glycosides in different durations of pretreatment (15, 30, 45 and 60 min) at 50°C, 1:5 (w/v) and 150 rpm.

Glycosides (mg/g)	15 (min)	30 (min)	45 (min)	60 (min)
Stevioside	15.85±0.27[a]	13.87±0.46[b]	13.14±0.82[b]	15.96±0.21[a]
Rebaudioside C	15.56±0.02[a]	14.97±0.08[b]	15.4±0.04[c]	19.62±0.71[b]
Rebaudioside A	27.57±0.23[a]	24.93±0.15[b]	24.97±0.05[b]	28.26±0.01[a]
Total glycoside extraction yield	58,98±0.54[a]	53,77±0.70[b]	53,51±0.77[b]	63,84±0.94[c]

[*] Means followed by lowercase in line do not differ ($P < 0.05$) statically between each other by (one-way ANOVA and Tukey test).

When the amounts (mg/g) of glucosides in the extract compared with the originally present on the leaf (Figure 1) are evaluated individually, it is assumed that AEUL loaded a higher stevioside content when compared with AEPL, however, in the two extracts there was a predominance of rebaudioside A. Individual analysis confirms AEPL, besides having a higher amount of glycosides, shows higher rebaudioside A and C content than AEUL, proven by ratio rebaudiosideA/stevioside (Table 3), however the enzyme pretreatment not only allow a greater recovery of the steviol glycosides, but also an improvement in the ratio RebA/Stevioside.

Erkucuk *et al.* (2009), performed a supercritical extraction with CO_2 and Soxhlet in Stevia leaves using

Table 3. Extraction yield (crude extract) and total glycosides in *Stevia rebaudiana* leaves without treatment and with enzymatic pretreatment.

Treatments	Ratio Reb A/Stevioside	Extraction yield of total glycoside (%)	Yield of aqueous crude extract (%)
AEUL	1.15	56.13 ±1.20[a]	24.1±0.01[a]
AEPL	1.75	71.39±0.38[b]	36.7±0.13[b]

[*] Means followed by same lowercase in column do not differ (P <0.05) statically between each other by (one-way ANOVA and Tukey test). Mean values of three determinations on each repetition±standard-error. AEUL: Aqueous extract untreated; AEPL: Aqueous extract with pretreatment.

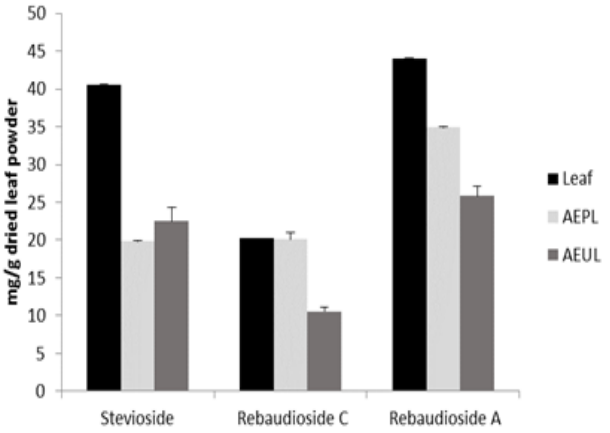

Figure 1. Stevioside, rebaudioside A and C content in aqueous extracts of pretreated leaves (AEPL) and of untreated leaves (AEUL) (1:10, 50°C, 3h) in one extraction cycle compared to the glycosides content present in leaves.

water and water:ethanol, respectively, as solvent, obtaining for supercritical with CO_2 41.10 mg/g of stevioside and 18.8 mg/g of rebaudioside A and for Soxhlet, 41.96 mg/g of stevioside and 22.53 mg/g of rebaudioside A. Abou-Arab *et al.* (2010), working with a hot water extraction at 65°C with ratio of 1:35 (w/v) in *S. rebaudiana* leaves during 3 hours, found maximum yield of 7.53 stevioside/100g of dry leaves. Whereas Periche *et al*. (2015), using conventional extraction technique, attained maximum yield at 90°C for 5 minutes, obtaining 22 mg/g of stevioside, 7 mg/g of rebaudioside A, 2.3 mg/g of rebaudioside C and 1 mg/g of dulcoside A.

Periche *et al.* (2015), previously mentioned, through modern techniques of extraction, using water as solvent, obtained maximum yields of steviol of 58.5 mg/g for ultrasonically and 72.1 mg/g for microwave assisted. Whereas in subcritical water extraction Yildiz-Ozturk *et al.* (2014) attained 74.35 mg/g. Yoda *et al.* (2003), in a supercritical extraction with CO_2 and water, from leaves with glycosides content of 10%, obtained maximum yield of 67% of glycosides originally present in the starting material. All compared values of recovery of steviol glycosides obtained with different extraction techniques (supercritical, subcritical, ultrasound and microwave-assisted) exhibited values similar to the ones obtained from the leaves enzymatic pretreatment (74.88 mg/g), showing that the methodology presented in this study is advantageous in relation to these other techniques which are expensive and may present application difficulties in industrial scale. Besides, a high extraction yield (366.84 mg/g) was calculated in just one extraction cycle, while most of the cited techniques requires a greater number of cycles to obtain the same level of yields obtained in the extraction with the use of enzymatic pre-treatment.

Conclusion

The pretreatment with cellulase enzyme before a conventional water extraction presented to be very effective for the extraction yield, since, when treated, there was a 65.6% increase in the yield of the crude extract. It was further observed an increase in recovery steviol glycosides, where 71% of the originally present in leaves were obtained when confronted to 53% of an extraction without treatment in one extraction cycle and among them, Rebaudioside A was obtained in higher content, which has superior sensory characteristics. It can also be said that the pretreatment duration directly influences the glycosides extraction yield, being the best time set to 1 hour. Then, as a viable alternative, there is the enzymatic pretreatment before the extraction of *S. rebaudiana* leaves, because it is a simple, fast and low cost methodology. It can be assumed the results of this study using *S. rebaudiana* leaves may go beyond this range of Stevia studied, however, it should be confirmed in further studies.

References

Atteh, J., Onagbesan, O., Tona, K., Buyse, J., Decuypere, E. and Geuns, J. 2011. Potential use of *Stevia rebaudiana* in animal feeds. Archivos de Zootecnia 60:133-136.

Abou-Arab, E. A., Abou-Arab, A. A. and Abu-Salem, M. F. 2010. Physico-chemical assesment of natural sweeteners stevioside produced from *Stevia Rebaudiana* bertoni plant. African Journal Food Science 4:269-281.

Barba, F. J., Grimi, N., Negm, M., Quilez F. and Vorobiev, E. 2014. Green recovery technology of sweeteners from *stevia rebaudiana* bertoni leaves. Leaf Sweeteners: Resources, Processing and Health Effects, January 01:41-55.

Dacome, A. S., Silva, C. C., Costa, C. E. M., Fontana, J. D., Adelmann, J. and Costa, S. C. 2005. Sweet

diterpenic glycosides balance of a new cultivar of *Stevia rebaudiana* (Bert.) Bertoni: isolation and quantitatative distribuition by chromatographic, spectroscopic and alectrophoretic methods. Process Biochemistry 40:3587-3594.

Erkucuk, A. Akgun, I. H. and Yesil-Celiktas, O. 2009. Supercritical CO_2 extraction of glycosides from *Stevia Rebaudiana* leaves: identification and optimization. Journal of Supercritical Fluids 5:89-35.

European Food Safety Authority (EFSA). 2011. Revised exposure assessment for steviol glycosides for the proposed uses as a food additive. European Food Safety Authority Journal 9(1):1972.

Gardana, C., Scaglianti, M. and Simonetti, P. 2010. Evaluation of steviol and its glycosides in *Stevia Rebaudiana* leaves and commercial sweetner by ultra high performace liquid chromatography-mass spectrometry. Journal Chromatography A 1217:1463-1470.

Ghanta, S., Banerjee, A., Poddar, A. and Chattopadhyay, S. 2007. Oxidative DNA damage preventive activity and antioxidant potential of *Stevia rebaudiana* (Bertoni), a natural sweetener. Journal of Agricultural Food Chemistry 55:10962–10967.

Kim, I-S., Yang, M., Lee, O-H. and Kang, S-N. 2011. The antioxidant activity and bioactive compound content of *Stevia rebaudiana* water extracts. Food Science and Technology 44:1328-1332.

Kroyer, G. 2010. Stevioside and Stevia-sweetener in food: application, stability and interaction with food ingredients. Journal of Consumer Protection and Food Safety 5(2):225-229.

Kovylyaeva, G. I., Bakaleinik, G. A., Strobykina, I. Yu., Gubskaya, V. I., Sharipova, R. R., Al'Fonsov, V. A., Kataev, V. E. and Tolstikov, A. G. 2007. Glycosides from *Stevia rebaudiana*. Chemistry of Natural Compounds 43(1):81-85.

Lemus-Mondaca, R., Vega-Gálvez, A., Zura-Bravo, L. and Ah-Hen, K. 2012. *Stevia rebaudiana* Bertoni, source of a high-potency natural sweetener: a comprehensive review on the biochemical, nutritional and functional aspects. Food Chemistry 132:1121–1132.

Liu J., Li J. W. and Tang J. 2010. Ultrasonically assisted extraction of total carbohydrates from *Stevia rebaudiana* Bertoni and identification of extracts. Food and Bioproducts Processing 88:215–221.

Pasquel, A., Meireles, M. A. A., Marques, M. O. M. and Petenate, A. J. 2000. Extraction of stevia glycosides with CO_2 + water, CO_2 + ethanol, and CO_2 + water + ethanol. Brazilian Journal of Chemical Engineering (Impress) 17(3):271-282.

Periche, A., Castelló, M. L., Herdia, A. and Escriche, I. 2015. Influence of Extraction Methods on the Yield of Steviol Glycosides and Antioxidants in *Stevia rebaudiana* Extracts. Plant Foods for Human Nutrition 70:119–127.

Pol, J., Hohnova, B. and Hyotylainen, T. 2007. Characterisation of *Stevia rebaudiana* by comprehensive two dimensional liquid chromatography time-of-flight mass spectrometry. Journal of Chromatography A 1150:85–92.

Puri, M., Sharma, D., Barrow, C. J. and Tiwary, A. K. 2012. Optimisation of novel method for the extraction of steviosides from *Stevia rebaudiana* leaves. Food Chemistry 132:1113–1120.

Soejarto, D. D. 2002. Botany of Stevia and Stevia rebaudiana. In: Kinghorn, A.D. (Ed.), *Stevia*: The genus *Stevia,* p. 18–39. Taylor and Francis, London and New York,

Prakash, I., DuBois, G. E., Clos, J. F., Wilkens, K. L. and Fosdick, L. E. 2008. Development of rebiana, a natural, non-caloric sweetener. Food and Chemical Toxicology 46:S75–S82.

Ray, C., Majumdar, G. C. and De, S. 2012. Optimization of process parameters for water extraction of stevioside using Response Surface Methodology. Separation Science and Technology 47:1014–1022.

Shukla, S., Mehta, A., Bajpai, V. K. and Shukla, S. 2009. In vitro antioxidante activity and total phenolic content of ethanolic leaf extract of *Stevia rebaudiana* (Bert.). Food and Chemical Toxicology 47:2338–2343.

Toma, M., Vinatoru, M., Paniwnyk, L. and Mason, T. J. 2011. Investigation of the effects of ultrasound on vegetal tissues during solvent extraction. Ultrasonics Sonochemistry 8:137–142.

Wang, J. X., Xiao, X. H. and Li, G. K. 2008. Study of vacuum microwaveassisted extraction of polyphenolic compounds and pigment from Chinese herbs. Journal of Chromatograph A 1198–1199:45–53.

Ye, F., Yang, R., Hua, X., Shen, Q., Zhao, W. and Zhang, W. 2013. Modification of stevioside using transglucosylation activity of *Bacillus amyloliquefaciens* α-amylase to reduce its bitter aftertaste. Food Science and technology 51:524-530.

Yildiz-Ozturk, E., Tag, O. and Yesil-Celiktas, O. 2014. Subcritical water extraction of steviol glycosides from *Stevia rebaudiana* leaves and characterization of the raffinate phase. The Journal of Supercritical Fluids 95:422–430.

Yoda, S. K., Marques, M. O. M., Petenate, A. J. and Meireles, M. A. 2003. Supercritical fluid extraction from *Stevia rebaudiana* Bertoni using CO_2 and CO_2 + water: extraction kinetics and identification of extracted components. Journal of Food Engineering 57:125–134