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Pretreatment with ethanol as an alternative to improve steviol glycosides extraction and purification from a new variety of stevia

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ABSTRACT

Leaves of a new variety of *Stevia rebaudiana* with a high content of rebaudioside A were pretreated with ethanol. The ethanolic extract showed high antioxidant potential and 39 compounds were identified, by UPLC/HRMS, among them one not yet mentioned in the literature for stevia leaves. From the *in natura* leaves and pretreated leaves, the conditions of aqueous extraction of steviol glycosides were investigated using response surface methodology. The aqueous extracts obtained were purified by ion exchange chromatography techniques and membrane separation methods. The recuperation of steviol glycosides was 4.02 g for pretreated leaves and 2.20 g for *in natura* leaves. The level of purity was, respectively, 87% and 84.8%. The results obtained demonstrate that pretreatment increases the yield and purity level of stevia sweeteners by the use of environmentally friendly methodologies and the final product presented acceptable sensory characteristics.

1. Introduction

Stevia rebaudiana (Bertoni) is a shrub that belongs to the Asteraceae family, which is native to Paraguayan regions and is also found in Brazil and Argentina (Lemus-Mondaca, Vega-Galvez, Zura-Bravo, & Ah-Hen, 2012). Its extracts are rich in steviol glycosides and are used as substitutes for sucrose (Yadav, Singh, Dhyani, & Ahuja, 2011).

In addition to the sweetening properties derived from steviol glycosides, which mainly include Stevioside, Rebaudioside A and C, studies have shown that extracts from its leaves also have other metabolites with bioactive potential, such as alkaloids, water soluble chlorophylls, xanthophylls, derivatives of hydroxycinnamoyl (derived from caffeine and chlorogenic acid), soluble oligosaccharides, free sugars, amino acids, lipids, essential oils and trace elements (Komissarenko, Derkach, Kovalyov, & Bublik, 1994), which are said to be responsible for producing therapeutic benefits, such as antihyperglycemic, antihypertensive, anti-inflammatory, antitumor, antidiarrheal, diuretic and immunomodulatory benefits (Chatsudthipong & Muanprasat, 2009).

When assessing the extraction and purification of steviol glycosides obtained from stevia leaves, we find a large body of literature involving conventional techniques and modern techniques (Jentzer, Alignan, Vaca-Garcia, Rigal, & Vilarem, 2015; Vanneste, Sotto, Courtin, et al., 2011; Yildiz-Ozturk, Tag, & Yesil-Celiktas, 2014). The extraction procedures involve solvents, enzymatic extractions, supercritical fluid methods, microwaves, ultrasound followed by purification involving columns, solvent-liquid-liquid extraction, ion exchange, ultra-membranes and nanofiltration, crystallization and fractional distillation. Despite the remarkable progress in extraction, the production of products with low impurity that are scalable using a minimum of solvents is still difficult (Rao, Reddy, Ernala, Sridhar, & Ravikumar, 2012)

Aiming to obtain sweeteners with acceptable sensorial characteristics using the minimal possible solvents during processing, the objective of this study was to achieve an ethanolic treatment of leaves of *Stevia rebaudiana*, to characterize the obtained byproduct and its potential as an additive in food, to investigate the changes generated by the treatment in the characteristics of the leaf and extraction yield and, finally, to define a methodology to obtain sweeteners and evaluate the sensorial characteristics of the final product.

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2. Methods

2.1. Sample and reagents

Leaves of *Stevia rebaudiana* were obtained at the experimental site for the Nucleus of Research in Natural Products (NEPRON) study located at the State University of Maringá. The leaves of the seminal variety, Stevia UEM-13, were in the flower bud formation stage (approximately 50–60 days after pruning) when the glycosides content of steviol was at a maximum. Afterward, they were dried in a forced circulation air oven until the humidity reached levels below 10%, crushed and stored.

All solvents and standards were LC grade or higher. Absolute ethanol (99.9%) by Merck (Londrina, Paraná, Brasil). Deionized water (18 M_{Ω}·cm) by Milli-Q plus system was purchased from Induslab (Londrina, Paraná, Brasil). All the reference compounds were provided by Sigma-Aldrich (Brasil).

2.2. Ethanolic pretreatment

The ethanolic treatment of *Stevia rebaudiana* leaves was performed by means of a column. Approximately 300 g of the leaves were packed with absolute ethanol. Afterward, the same solvent was eluted using gravity and 14 ethanolic fractions of 350 ml were obtained. Afterward, the leaves and fractions were dried and stored for subsequently obtaining the sweeteners and characterizing the fractions.

2.3. Characterization of leaves

To investigate whether the ethanolic treatment generated changes in leaf characteristics, the composition was analyzed before and after treatment.

2.3.1. Physico-chemical analysis

Moisture and ashes were measured according to the methodology proposed by the Adolfo Lutz Institute (2005). For Chlorophyll *A* and *B*, the Arnon method (1949) was used, and for anthocyanins, the method described by Lee and Francis (1972) was used.

2.3.2. Fatty acid extraction

Stevia rebaudiana leaves were extracted to obtain total fatty acids with low toxicity solvents as described by Biondo et al. (2015) with some adaptations. Ethanol:hexane:water (5:5:2) was used as the solvent, and after separation of phases with a separation funnel, the hexane phase was dried to obtain the oil and for subsequent characterization of the fatty acids.

2.3.3. GC–MS

For the quantification of fatty acids extracted from stevia leaves, esterification was performed as described by Santos Júnior et al. (2014). The fatty acids methyl esters (FAME) were separated in a gas chromatographer, Thermo, Trace Ultra, equipped with a flame ionization detector and fused silica capillary column CP – 7420 (Select FAME, 100 m in length, 0.25 mm in internal diameter, and 0.25 μm of cyanopropyl). The exhaust gas flows were 1.2 ml min^{-1} for the carrier gas (H_2), 30 ml min^{-1} for the make-up gas (N_2), 35 and 350 ml min^{-1} for the H_2 and the synthetic air, respectively, for the flame of the detector. The injected volume was 2.0 μl, using a split sample of 1:80. The temperatures of the injector and detector were 240 °C. A temperature of 165 °C was used in the column, for 7.00 min, followed by a heating ramp of 4 °C min^{-1} until reaching 185 °C, remaining in this condition for 3 min, followed by a new heating ramp of 6 °C min^{-1} until the column reached 235 °C, for 1.67 min, totaling therefore 25 min of analysis. The times of retention and the FAME peak areas were determined using the software ChromQuest™ 5.0.

2.4. Experimental design

Before obtaining the sweetener as a powder, a study of the conditions of extraction was conducted at the central point. It contained three variables and two levels, totaling three replicates at the central point with 11 experiments. Response values are expressed as total steviol glycosides extraction yield (mg/g dry leaf).

To evaluate the total yield of steviol glycoside extraction (Y) as a function of the three independent variables time (x_1), temperature (x_2) and leaf:water ratio (x_3).

2.5. Obtaining sweetener powder

After studying the influence of the processing variables on the total yield of steviol glycosides, the ideal extraction conditions were defined. A methodology was proposed to obtain the sweetener where the minimum amount of solvents was used. For this, *Stevia rebaudiana* leaves were extracted with water (1:27) w/v at 60 °C and 200 rpm in three cycles. Then, the crude extract was filtered under a vacuum to remove the suspended particles. For purification, the filtered crude extract passed through a UF (10 kDa) and NF (500 Da) membrane with an ion exchange and adsorption column using as eluant ethanol: water (85:15) v/v. Finally, the material was dried in a rotary evaporator, and the purified, powdered sweetener was obtained. At the end of each step of the process, an aliquot was collected to investigate the loss of compounds.

2.6. Quantification of steviol glycosides by HPLC

The total glycosides present in the leaves, in the ethanolic extracts obtained in the treatment column, in the fractions removed during each purification step of the sweetener and in the final powdered product were identified and quantified by High Performance Liquid Chromatography (HPLC) coupled to an index detector refraction S:32 with a 5 μm NH2 column 125 × 4.6 mm in size using acetonitrile: water (80:20) v/v as the mobile phase with HPLC grade.

2.7. Identification of compounds, bioactive compounds and antioxidant activity in vitro

The ethanolic fractions and aqueous extract were analyzed (1 mg/ml) to evaluate the bioactive potential and its possible use as a by-product, as well as for the identification of these compounds. The aliquots collected after the aqueous extraction process were also investigated.

2.7.1. Total phenolic compounds

Total phenolic compounds were determined according to the method described by Singleton, Orthofer, and Lamuela-Raventos (1999). The absorbance was measured at 760 nm. The total phenolic concentration was expressed as mg of gallic acid equivalent (EAQ)/mg extract.

2.7.2. Flavonoids

The quantification of total flavonoids was performed using the method described by Jia, Tang, & Wu, 1999. All extracts and fractions were prepared at a concentration of 1 mg/ml of ethanol, except for the ethyl acetate fraction (0.5 mg/ml). The absorbance of the samples was measured at 510 nm. Data were expressed as quercetin equivalents.

2.7.3. Antioxidant actives

The free radical scavenging activity of extracts and aliquots was measured by the ability to eliminate DPPH radicals (Blios, 1958). The absorbance was measured at 517 nm, and gallic acid was used as the reference compound. The results were expressed as percent inhibition of free radicals.