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Development and optimization of acaloric sweeteners extraction procedure from *Stevia rebaudiana* Bertoni

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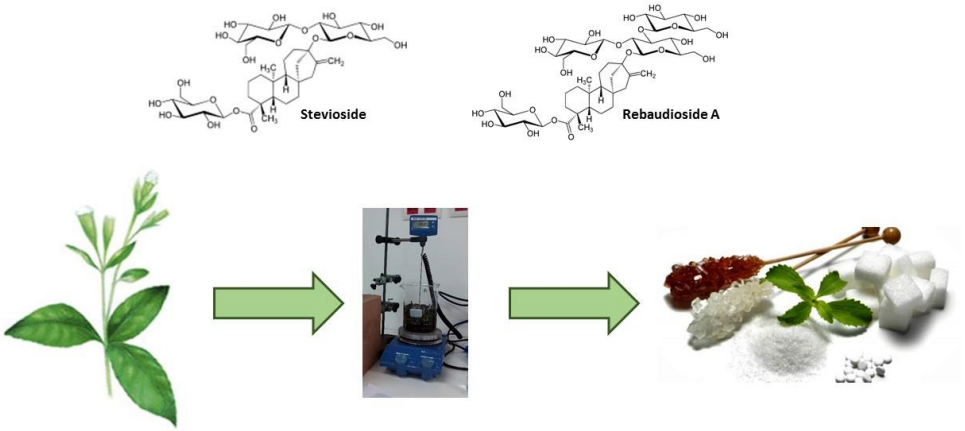
Abstract: *Stevia rebaudiana Bertoni is a shrub of the Asteraceae family native to South America, especially of Brazil and Paraguay. This plant accumulates a suite of diterpenoid metabolites that are natural sweeteners finding increased use as sugar. In addition, a number of healing properties have been associated to the stevia leaves intake. The objectives of the present work have been to develop an optimum extraction method of the main steviol glycosides, stevioside and rebaudioside A, from the Stevia leaves studying the influence of the several factors that affect the process. Applying experimental design methodology, parameters such as, the grinding of the leaves, the temperature and time of extraction, the agitation of the process, the quantity of dried leaves and the volume of dissolvent were assessed. In order to eliminate the impurities and to obtain a crystalline white precipitate several procedures were tested. Moreover, a HILIC chromatographic method to identify and quantify steviol glycosides from stevia leaves were optimized.*

Keywords: *Stevia rebaudiana; stevioside; rebaudioside A; HILIC*

Graphical Abstract:



Introduction:

The increasing consumption of sucrose has resulted in several nutritional and medicinal problems, including obesity[1]. Nowadays, the most common high intensity sweeteners in the world market are made of synthetic compounds which are toxics when they are used in excess[2]. On the other hand, numerous sweet and low caloric compounds are available in nature. *Stevia rebaudiana* Bertoni is a perennial shrub of the Asteracea family, native to the Amambay and Iguaçu districts on the borders of Brazil and Paraguay[3]. It is about three hundred times sweetener than sugar[4]. In addition to their sweetening properties, some studies suggest that Stevia extracts have therapeutic properties[3,5,6].

The major compounds responsible for sweet taste of *Stevia* are the diterpenoid glycosides stevioside (4-13% w/w), rebaudioside A (2-4% w/w), rebaudioside C (1-2% w/w) and dulcoside A (0.4-0.7% w/w)[7]. These compounds, which are known as Stevia sweeteners, are the glycosides of the diterpene steviol ent-13-hidroxikaur-16-en-oico acid[8]. The other glycosides present in lower concentrations are steviolbioside, rebaudioside B, rebaudioside D, rebaudioside E and rebaudioside F [9].

The aim of this work was to develop an optimum extraction method of the main steviol glycosides, stevioside and rebaudioside A, from the Stevia leaves studying the influence of the several factors that affect the process using experimental design methodology. The quantification has been carried out by HPLC-DAD. Several procedures were tested for the extract clarification.

Materials and Methods:

Samples

Samples were prepared from Stevia leaves supplied by Axarquía (Spain). The leaves are sold dry and whole. They were grinded according to the requirements of the different tests carried out.

Reagents and standards

Tap water was used for the different extraction procedures. HPLC-grade acetonitrile was used from Fisher Chemical (EEUU). Deionized water (18MΩ cm) was produced using a Milli-Q water purification system from Millipore (Bedford, MA, USA).

Stevioside and rebaudioside A with purities (≥99.0%) were purchased from Sigma-Aldrich (Steinheim, Germany). The calibration solutions for the HPLC analysis of stevioside and rebaudioside A were prepared daily by diluting the analytes with acetonitrile/water (8:2 v/v).

Extraction conditions

To choose the best extraction conditions for the steviol glysosides, we tried both milled and unmilled dry leaves. Other factors such as the temperature and time of extraction, agitation of the process, the quantity of dried Stevia leaves

and the volume of solvent were studied using experimental design methodology.

The procedure carried out for the extraction of stevioside and rebaudioside A was based on heating a given volume of water at a given temperature and casting it on a heavy amount of *Stevia Rebaudiana* dry leaf according to the values indicated by the experimental design assays. The extraction procedures were repeated twice. The solutions were rapid cooled to room temperature using ice water. After the extracts were cooled, the leaf residues were removed and the extract was filtered through a 0.45 μm membrane (Millipore) followed by a 1000 Da membrane (Sartorius).

One millilitre of the extract was diluted to 10 ml with acetonitrile/water (8:2 v/v). This solution was filtered through a membrane filter (0.22 μm) before the HPLC analysis. All the samples were analysing in triplicate.

Determination of steviol glycosides

The analysis was carried out with an Agilent 1200 HPLC with DAD detector (Agilent Technology, Palo Alto, CA, USA). Phenomenex Hilic column (50x2.1 mm, 1,7 μm particle size) thermostated at 25º C, with gradient elution mode, was used in order to determine the compounds studied. Different ramps of deionized water (eluent A) and acetonitrile (eluent B) were tested. The optimum gradient was as follows: start with 90% B, change from 90% to 80% in 4 min, change from 80% to 70% in 6 min and constant 90% B for 9 min more. A post-run of 3 min was programmed to equilibrate the column between analyses. The flow rate was 0.2 mL/min, and the sample injection volume was 3μl. The DAD detector was set at 200 and 210 nm. Steviol glycosides were identified by comparison with the retention time and the spectra of their pure standards.

Purification of extracts

In order to eliminate the impurities and to obtain a crystalline white precipitate several procedures were tested, being the solid phase extraction with NH_2 cartridges the only procedure that allowed to obtain clean extracts.

Results and Discussion:

The optimization of the steviol glycosides extraction was carried out by a factorial screening design of five variables at two levels (2^{n-1}) with a V resolution. The variable parameters were the grinding of the leaves, the temperature and time of extraction, the agitation of the process, the quantity of dried Stevia leaves and the volume of dissolvent. The experiments were performed in random order to avoid the introduction of systematic errors. The extracts used in these tests were obtained following the procedure described in the experimental section. The brix degrees and stevioside and rebaudioside A contents were the dependent variables used for this analysis. Data obtained were treated by a statistical program (StatSoft, Tulsa, USA). The results showed that time and agitation did not present significant effects on any of the dependent variables.

In order to find the optimal values of the factors which presented significant effects on the dependent variables, a three-factor "Box-Behnken" experimental design was carried out keeping the time of extraction and the agitation of the process constant. The results acquired were analyzed. The response surfaces obtained are shown in Fig.1, 2 and 3.

Fig. 1 shows the evolution of the brix degrees according to the grinding of the leaves and the ratio (the quantity of dried Stevia leaves and the volume of dissolvent). As it can be seen on the brix degrees increased with increasing the two variables. This means that both the leaves grinding and the ratio had a strong effect on the brix degrees.

Fig. 2 illustrates the evolution of the stevioside concentration according to the grinding of the leaves and the ratio. As it can be observed from the graph, the stevioside concentration increased with increasing the variables, but from a medium degree of grinding, the content of stevioside lowers, so the optimum grinding value will be an intermediate value.

Fig. 3 shows the evolution of the rebaudioside A concentration according to the grinding of the leaves and the temperature (Fig. 3a), according to the ratio and the temperature (Fig. 3b) and according to the ratio and the leaving grinding (Fig. 3c) with the agitation and the time constants in all cases. As it can be seen from the graph, the

rebaudioside concentration increased with increasing the variables, but from a medium degree of grinding, the content of rebaudioside A also lowers, so the optimum grinding value will be an intermediate value.

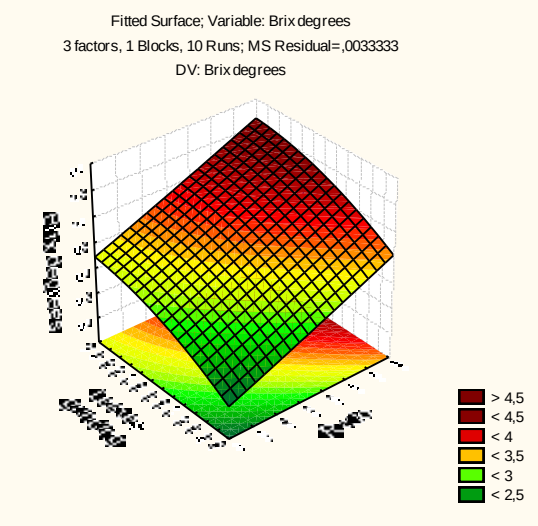


Fig. 1. *Response surfaces plot for brix degrees showing the effect of the leaves grinding and the ratio with the time of extraction and the agitation of the process constant.*

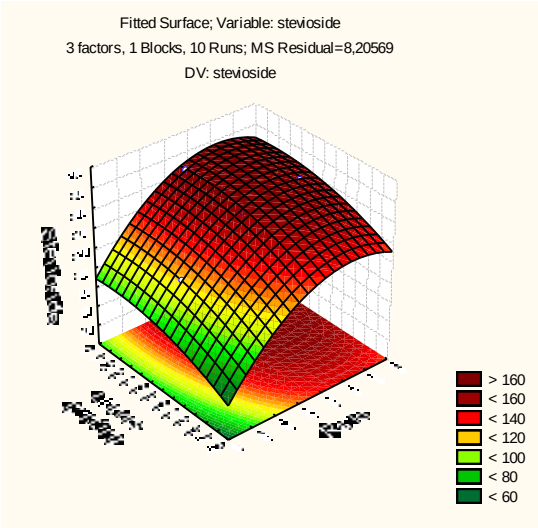


Fig. 2. *Response surfaces plot for stevioside concentration showing the effect of the leaves grinding and the ratio with the time of extraction and the agitation of the process constant.*

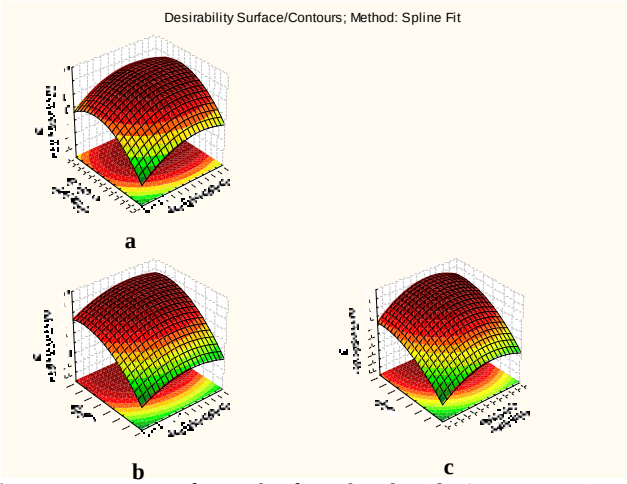


Fig. 3. *Response surfaces plot for rebaudioside A concentration showing the effect of the leaves grinding and the temperature (a), the ratio and the temperature (b) and the ratio and the leaving grinding (c) with the time of extraction and the agitation of the process constant in all cases.*

According to the results obtained in both experimental designs, we have chosen as optimal values the following: 100 g/L for leaf-water ratio, degree of intermediate grinding, a temperature of 75 °C and without agitation for 20 minutes.

Conclusions:

An extraction process has been developed and optimized for obtaining glycosides from the plant *Stevia rebaudiana Bertoni*. The extract obtained by the different experiments was characterized chromatographically with the idea of quantifying the response.

The screening experimental design revealed that the significant variables in the extraction process were: the leaf-water ratio, temperature and degree of grinding and the period of time and the agitation did not affect significantly the process. They were fixed in 20 minutes without stirring for subsequent assays. The analysis of response surfaces, showed that the optimal values were 100 g/L, for leaf-water ratio, degree of intermediate grinding and a temperature of 75° C.

The optimal results for purification of the extracts obtained with NH_2 cartridges of solid phase extraction, allowing the pigment retention and the elution of the glycosides of interest.

For all these reasons, the proposed methodology is adequate for determining to the major steviol glycosides in a quick and easy procedure, and

with a lower environmental impact than other methodologies described in literature.

Conflicts of Interest:
The author declares no conflict of interest.

References:

1. Anton. S.D.; Martin, C.K.; Han, H.; Coulon, S.; Cefalu, W.T.; Geiselman, P.; Williamson, D.A. Effects of *Stevia*, aspartame, and sucrose on food intake, satiety, and postprandial glucose and insulin levels. *Appetite* **2010**, 55(1), 37-43.
2. Martínez Pérez, T. La diabetes y su control con Stevia. *Libros en red* **2004**.
3. Puri, M.; Sharma, D.; Barrow, C.J. Enzyme assisted extraction of bioactive from plants. *Trends in Biotechnology* **2011**. (doi:10.1016/j.tibtech.2011.03.014).
4. Geuns, J.M.C.; Augustijns, P.; Mols, R.; Buyse, J.G.; Driessen, B. Metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. *Food Chem. Toxicol*. **2003**, 41 (11), 1599-1607.
5. Chatsudthipong, V.; Muanprasat, C. Stevioside and related compounds: 808 Therapeutic benefits beyond sweetness. *Pharmacology & Therapeutics* **2009**, 121(41-809), 54.
6. Sharma, D.; Puri, M.; Tiwary, A.; Singh, N.; Jaggi, A. Anti-amnesic effect of Stevioside in scopolamine treated rats. *Indian Journal of Pharmacology* **2010**, 42, 164-167.
7. Geuns, J.M.C. *Phytochemistry* **2003**, 64, 913.
8. Prakash Chaturvedula, V.S.; Upreti, M.; Prakash, I. Diterpene glycosides from *Stevia rebaudiana*. Molecules **2011**, 16 (5), 3552-3562
9. Starratt, A.N.; Kirby, C.W.; Pocs, R.; Brandle, J.E. *Phytochemistry* **2002**, 59, 367.