DETERMINATION OF GLYCOSIDES, IN EXPRESS HONEY OF STEVIA (*Stevia rebaudiana*) BY LIQUID CHROMATOGRAPHY

DETERMINACIÓN DE GLÍCOSIDOS, EN MIEL EXPRESS DE ESTEVIA (*Stevia rebaudianai*) POR CROMATOGRAFÍA LÍQUIDA

DETERMINAÇÃO E QUANTIFICAÇÃO DE GLICOSÍDEOS, NO MEL EXPRESSO DAS ESTÉVIA (*Stevia rebaudiana*) POR CROMATOGRAFIA LÍQUIDA

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ABSTRACT

The aim of this study was to incorporate naturally into honey the main sweet diterpene glycosides found in Stevia rebaudiana, called stevioside and rebaudioside A, in order to combine the sweetening properties of these two substances. To determine their degree of incorporation, the diterpene glycosides were identified and quantified both in the prepared syrups and in the honey obtained by the analytical technique of high performance liquid chromatography (HPLC) using a NH_2 column (Zorbax-Agilent) and, as a mobile phase, a mixture of acetonitrile: water (70:30) with ultraviolet detection to 194 nm. Quantification was performed by means of a calibration curve where high rates of incorporation, exceeding 97% for stevioside and rebaudioside A, were found. For the standardization of the analytical technique, parameters were determined such as linearity, analytical sensitivity, detection limit, quantification limit and precision, showing that the method developed is

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simple, fast and reliable within the established limits, and levels up to 0,08 ppm for stevioside and 0,09 ppm for rebaudioside A.

RESUMEN

El objetivo del presente trabajo fue incorporar de forma natural en la miel los principales glicósido diterpenos dulces de la Stevia rebaudiana, denominados esteviósido y rebaudiósido A, con el fin de reunir las propiedades edulcorantes de estas dos sustancias; para comprobar su grado de incorporación se identificaron y cuantificaron los glicósido diterpenos tanto en los jarabes preparados como en la miel obtenida mediante la técnica analítica de cromatografía líquida de alta resolución (HPLC) usando una columna NH_2 (Zorbax-Agilent) y como fase móvil una mezcla de acetonitrilo:agua (70:30) con detección en el rango ultravioleta a 194 nm. La cuantificación fue realizada por medio de una curva de calibración encontrándose porcentajes de incorporación altos, superando el 97 % para esteviósido y rebaudiósido A; para la estandarización de la técnica analítica se determinaron parámetros como: linealidad, sensibilidad analítica, límite de detección, límite de cuantificación y precisión, demostrándose que el método desarrollado es simple, rápido y confiable, dentro de los límites establecidos, se detectaron niveles hasta de 0,08 ppm para steviósdio y 0,09 ppm para rebaudiósido A.

RESUMO

O objetivo deste estudo foi o de incorporar um doce mel natural diterpenes as principais glicósido de Stevia rebaudiana, estévia e apelou rebaudiósido A, a fim de satisfazer as propriedades edulcorantes destas duas substâncias, a fim de verificar seu grau de incorporação foram identificados glicósido diterpenes quantitated e em preparações, como xaropes de mel obtidas pela técnica analítica de cromatografia líquida de alta eficiência (CLAE), utilizando uma coluna NH_2 (Zorbax, Agilent) como fase móvel e uma mistura de acetonitrila: água (70:30) com detecção no ultravioleta gama em 194 nm. A quantificação foi realizada por meio de uma curva de calibração foi encontrado altas taxas de incorporação, superior a 97% para estéviosido e rebaudiosido A; para a padronização dos parâmetros analíticos foram determinados: linearidade, sensibilidade analítica, de detecção, limite identificação e precisão, mostrando que o método desenvolvido é simples, rápido e confiável dentro dos limites foram detectados níveis de até 0,08 ppm e 0,09 ppm para steviósdio para rebaudiósido A.

KEYWORDS:
Stevioside, Rebaudioside A, HPLC, Quantification.

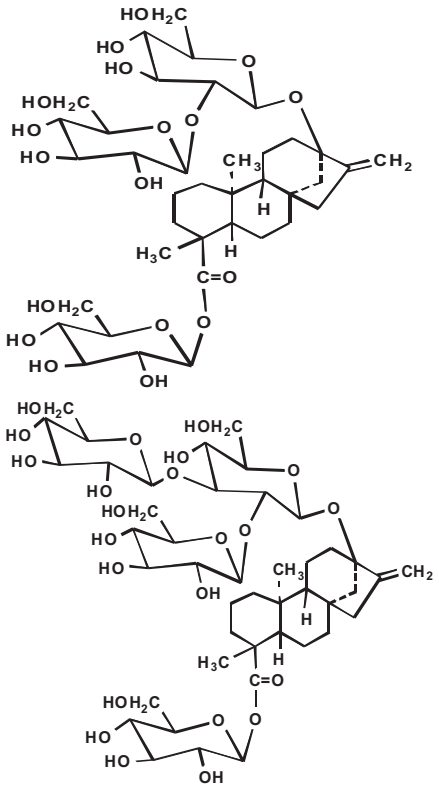
PALABRAS CLAVES:
Steviósido, Rebaudiósido A, HPLC, Cuantificación.

PALAVRAS-CHAVE:
Steviósido, Rebaudiósido A, HPLC, Quantificação rápida.

INTRODUCTION

Stevia rebaudiana Bertoni, a Paraguayan herb also known as Kaa Hee or "sweet herb", has attracted a lot of interest as a potential source of natural non-caloric sweetener for use as a substitute for synthetic sweeteners. This plant is being used as a great deal in Brazil, Argentina, Paraguay, China, Korea and Japan. Stevia rebaudiana contains six diterpene glycosides with a strong sweet taste, the most abundant and important of which is ste-

Figure 1. Structure of stevioside and rebaudioside A



vioside, with a sweetening power 300 times greater than sucrose. Rebaubioside A is second in abundance and is 400 times sweeter than sucrose. The rest is of less commercial interest: rebaudioside C and dulcoside A and at trace level, rebaudioside E and D [1-3]. It has been shown that stevioside directly stimulates pancreatic B cells to considerable insulin secretion, so it is ideal for diabetics [4-8]. They are commonly used as natural sweeteners in beverages and foods [13-15], with their main properties including thermal stability up to 238ºC, resistance to acid hydrolysis, they are not fermentable and, most importantly, non-toxic, making them quite attractive as additives in food and clinical applications [16-19]. Figure 1 presents their structures.

In this case Stevioside and Rebaudioside A are used for the preparation of syrups in the food of bees, especially in winter time or when flowering is scarce. In the Alps Apiary of the company Productos Naturales Arroyave, located in the Department of Cauca (Colombia), studies have been undertaken feeding bees with fruit juices and commercial sugar, from which honeys have been produced which are known commercially as honey express or frutimiel. There has been success with express honeys such as soybean, eucalyptus, milk, guava, passionfruit and Stevia. The main feature of this product is the combination of the properties of the honey and the juice added [20]. A wide range of analytical techniques have been used to identify and/or quantify the steviosides in foods, drinks and different matrices [21-24] which include TLC [26], capillary electrophoresis, enzymatic and infrared spectroscopy techniques. The most common analytical method is high pressure liquid chromatography (HPLC) [27-30] in which the detection is performed using the UV-Vis detector, and the separations using columns such as C_{18}, Hidroxiapatite and amino.

The aim of this work is to obtain a honey which contains greater sweetening power than natural honey, called express honey from Stevia, besides achieving the standardization of the HPLC methodology for the identification and quantification of stevioside and rebaudioside A present in this honey.

METHOD

This research was conducted in two stages, the first in the field and the second in the laboratory.

Fieldwork

The fieldwork was carried out following the methodology described by Benítez *et all. to obtain express honey, with selected hives.* [31]

Selection of the hives. We worked with bees of genus *Apis mellifera*, using six large hives.

Preparation of the syrups. All of the required sugar was dissolved in boiling water, and then Stevia extract was added and the mixture was allowed to boil again

Table 1. Formulations for the preparation of Stevia-based syrups and sugar trade.

Formulation		
Ingredients	**Double strength sweetness**	**Triple strength sweetness**
Kg of water	1,000	1,000
Kg of sugar	1,300	1,300
Kg of Stevia extract	0,005	0,010
Hives	1, 2, 3	4, 5, 6

for 5 minutes. Table 1 shows the amounts of each of the ingredients.

The dates according to the calendar to provide food for the bees are associated with periods of rain or lack of bloom throughout the year.

Express Honey Harvest. The harvest of express honey was made when there were cells of sealed honey, using the same criteria as for normal honey, following the methodology described by Benítez *et all.* [31]

Working in the laboratory

Samples were taken to the laboratory for analysis as indicated below

Chromatographic analysis. We used a high performance Hewlett-Packard 1100 Liquid chromatograph, UV-Vis detector, analytical column Agilent Zorbax NH_2- 5μm x 4,6 x 250 mm, wavelength 194 nm, mobile phase acetonitrile:water (70:30) in isocratic mode, with flow of 0,6 mL/min. Conditions developed in the QPN laboratory.

Stevioside and rebaudioside A ITI Miami International Inc., Florida. standards were used. The Stevia extract was purchased from Bio-Stevia SA©, Santiago de Cali-Colombia).

Sample preparation. 0,020 g of Stevia honey was weighed, diluted to 10,0 mL, and passed through a nylon filter of 0,45 μm for identification by HPLC .

RESULTS

The retention times for Stevioside and rebaudioside A were determined in the HPLC column, it can be seen in the table 2. In the chromatographic profile, in figure 2, it is shown that these two glycosides can be separated with a resolution to baseline level under the experimental conditions described above.

Calibration curve

The coefficients of determination (R^2) and the equation of the curve, it can be seen in the table 2, were calculated yielding R^2 values very close to 1,0, indicating that the curve presents a linear behavior and is directly proportional, with a low dispersion of values.

Accuracy (Repeatability). To determine the accuracy of the method the area of two standard solutions of stevioside and rebaudioside A of 40 ppm was measured ten times consecutively and standard deviations were calculated based on the average area, in order to assess repeatability. According to the results obtained, one can observe that the values of RSD, under a parameter of confidence of 95% are low, so the method for quantification of stevioside and rebaudioside A by HPLC showed repeatability. It can be seen in the table 2.

Reproducibility. To determine the reproducibility of the method, a standard solution of 10 ppm concentration of the two glicosides was used, the measurements were carried out in triplicate for thirty days, with values less than 1,5% relative standard deviation (RSD) indicating that the chromatographic method is reproducible and reliable, it can be seen in the table 2.

Figure 2. Chromatographic profile of the standards for stevioside and rebaudioside A

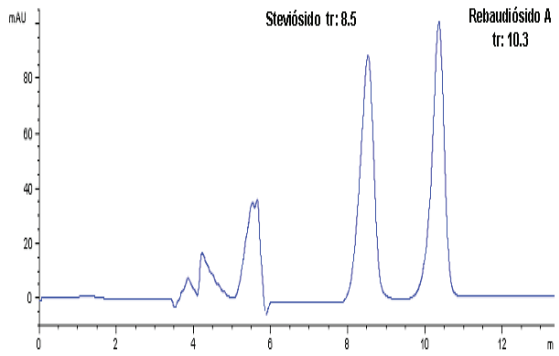


Table 2. Parameters found in the standardization of the method

Parameter	Stevioside	Rebaudoside A
Retention time (tr) (min)	8,5	10,3
R^2	0,9984	0,9982
Curve equation	Y=8,998x + 5,225	Y=18,643x - 2,387
RSD (repeatability) (%)	1,34	0,25
RSD (reproducibility) (%)	0,41	0,74
Recovery rate (%)	99,58	99,11
LD detection limit (mg/L)	0,08	0,09
LQ Quantification limit (mg/L)	0,28	0,32

Accuracy. The recovery by this method is high surpassing 99% for stevioside and rebaudioside A, thus verifying the accuracy of the system, It can be seen in the table 2.

Detection limit and quantification limit. To assess the sensitivity of the instrument and to calculate the minimum concentration detected of stevioside and rebaudioside A, two additional calibration curves are constructed in a concentration range of 1-5 mg/Kg for these glycosides. The values of both the detection limit and quantification limit are quite low indicating that the proposed methodology is sensitive and the UV detector is ideal because it has a good sensitivity to generate a minimum signal of these compounds, it can be seen in the table.

The table 3 presents the percentage of incorporation of stevioside and rebaudioside A found in honey samples with double and triple Stevia sweetening power for the six hives evaluated, an amount which exceeds 97% for stevioside and rebaudioside A. In figure 3 the chromatogram is shown for a sample of Stevia honey.

With these results, it was shown that it was possible to incorporate the main glycosides of Stevia into the honey. In order to determine whether the increased sweetness present in Stevia honey was detected by consumers, a sensory evaluation was performed.

By carrying out this study it could be determined that the bees properly assimilated the main glycosides of the Stevia, which was shown by high percentages of incorporation exceeding 97% for stevioside and rebaudioside A.

CONCLUSIONS

This research and the results obtained in it have allowed us to find a new way of producing express honey, obtaining a product with added value and features that make it attractive to the different national markets and with a high profile international. The high performance liquid chromatography (HPLC) with UV detection proved to be a good technique and a useful tool for confirmation of the existence of stevioside and rebaudioside A in samples of honey and Stevia and for quantitative determination. We managed to optimize the chromatographic parameters such as linearity in the working range, precision, accuracy and sensitivity of the method for standardization of analytical methodology that allowed the determination of the presence of stevioside and rebaudioside A in Stevia honey samples efficiently and reliably.

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Figure 3. Chromatograms for a sample of Stevia honey.

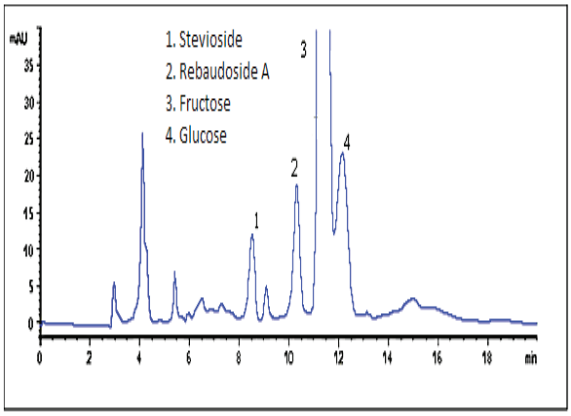

Table 3. Amount of stevioside and rebaudioside A found in honey samples with double and triple Stevia sweetening power (DPE and TPE)

Hive	Concentration	Syrup (mg/g sample)		Stevia Honey (mg/g sample)		% Incorporation Stevioside	% Incorporation Rebaudioside A
		Stevioside Concentration	Stevioside Concentration	Stevioside Concentration	Rebaudioside A Concentration		
1	DPE	1,44	3,12	1,41	3,06	97,9	97,4
2		1,39	3,07	1,35	3,05	97,1	99,4
3		1,31	3,18	1,29	3,14	98,5	98,7
4	TPE	2,87	6,11	2,79	5,90	97,2	96,6
5		2,77	6,08	2,72	5,90	98,2	97,0
6		2,62	6,00	2,57	5,78	98,1	96,8

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