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Determination of steviol glycosides in seven *Stevia rebaudiana* (Bertoni) extracts routinely used in the food and confectionary industry

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ABSTRACT

Stevia rebaudiana Bertoni extracts are used as natural non-nutritive sweeteners in several countries worldwide. The principal components of stevia leaf extracts are stevioside and rebaudioside A. Stevioside has about 300 times the sweetening power of sucrose, but is the main cause of the bitter aftertaste of crude stevia extracts. Rebaudioside A has greater sweetening power and a less pronounced bitter aftertaste, and so is usually the preferred component. The overall taste of the sweetener depends on the composition of the stevia extract used and the steviol glycosides present. To characterize the unique taste profile of stevia sweeteners, we analyzed stevioside, rebaudioside A, rebaudioside B and isosteviol content in seven different commercial food-grade extracts used as raw material. A sensitive method, using HPLC coupled to electrospray ionization-tandem mass spectrometry, was applied. The amount of steviol glycoside in the seven different extracts varied. As expected, rebaudioside A was the most abundant, ranging from 497 to 1,000 mg/g. One sample contained only rebaudioside A. Rebaudioside B and stevioside levels ranged from negligible to 40% of the total glycosides determined, with concentrations ranging from 2.7 to 386 mg/g of raw material. The concentration of isosteviol was below the limit of detection in all samples. These data can help in the selection of the most suitable commercial extracts for use as sweeteners in food, beverages and confectionery.

Keywords
Sweetener
Stevia rebaudiana
Steviol glycosides
ESI-MS/MS
Food
Beverage
Confectionery manufacture

Introduction

In light of the high incidence of obesity and diabetes worldwide, food and confectionery manufacturers have a duty to help reduce calorie intake by lowering the amount of sugar present in beverages and food. Substitution of sugar with natural and artificial low-calorie sweeteners meets the increasing demand for low-sugar products by consumers and is thus commercially desirable.

Among the low-calorie sweeteners, *Stevia rebaudiana* extracts have received increasing attention from manufacturers in recent years. *S. rebaudiana* Bertoni, a herbaceous plant of South American origin, is a natural non-nutritive sweetener that has been used for centuries by local populations and is now being used to sweeten foods and beverages in other countries [1]. The strong sweetening effect of *S. rebaudiana* is due to the presence of diterpenoid glycosides in the leaves, most of which derive from the aglycone steviol. The main glycosides are stevioside and rebaudioside A, which account for 10% and 2–4%, respectively, of leaf dry weight. Several other steviol glycosides are present in lesser amounts up to 1–2%, including a minor component from the steviol aglycone isomer, namely isosteviol [2, 3].

In addition to their sweetening properties, steviol glycosides have been reported to exert hypotensive effects [4], to have a beneficial hypoglycaemic role in an animal model of type 2 diabetes [5] and to reduce postprandial blood glucose in patients with type 2 diabetes [6]. Steviol glycosides are non-cariogenic sweeteners [7] and stevia leaf extracts are a source of antioxidant compounds [8, 9].

All steviol glycosides have the same metabolic fate and are transformed both in vitro and in vivo to steviol. In vitro and ex vivo studies have confirmed that ingested steviol glycosides are not hydrolyzed by the digestive enzymes of the upper gastrointestinal tract, are not adsorbed, and enter the colon where they are metabolized to steviol by the intestinal flora [10]. Additionally, there is an efficient phase II detoxification process in humans that quickly converts steviol to its glucoronide, with rapid urinary excretion [1].

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Although steviol glycosides and steviol exerted weak mutagenic activity in the highly sensitive *Salmonella typhimurium*, carcinogenicity and genotoxicity studies indicated there was no substantial risk associated with stevia extracts. Thus, their use as a sweetener should not cause consumer concern [1, 11].

The safety of some steviol glycosides has been extensively evaluated and they have been authorized for use as food additives in several countries including Argentina, Brazil, Paraguay, South Korea and Japan [12]. High-purity steviol glycosides (≥95%), including stevioside and rebaudioside A, and a limited number of other stevia components, are now Generally Recognized as Safe (GRAS) by European [13, 14] and American [15] agencies and are commonly used as sweeteners in foodstuffs and confectionery and are sold to consumers as a sugar substitute [10, 16].

With their good safety profile, 11 steviol glycosides (stevioside, rebaudiosides A, B, C, D, E, F and M, steviolbioside, rubusoside and dulcoside) found in stevia leaf extracts are now certified by international regulatory agencies [13, 14]. Rebaudioside B and isosteviol may also be present in stevia extracts as by-products formed during manufacture [17].

Each steviol glycoside contributes its own unique taste profile to the overall taste of the sweetener. Crude extracts of *S. rebaudiana* may have a bitter or liquorice-like aftertaste which can affect the quality of the final product. Stevioside, the most abundant glycoside in *S. rebaudiana* leaves, has about 300 times the sweetening power of sucrose, but is also the main cause of the bitter aftertaste of crude stevia extracts. Rebaudioside A, although there is less of it, has greater sweetening power and a less pronounced bitter aftertaste, so it is usually the preferred component in stevia leaf extracts. The overall taste of the sweetener depends on the composition of the stevia extract used, while the steviol glycoside composition of each extract depends on genetics, geographical origin, growing conditions and the extraction procedure.

Quality control procedures are important to confirm the composition of the raw materials used in the food industry to ensure that the standardization and safety requirements set by regulatory agencies are met. Such procedures also ensure that the most suitable extracts containing the required steviol glycosides are selected.

A number of different techniques, including high-pressure liquid chromatography (HPLC) [18, 19] and HPLC-tandem mass spectrometry, have been described for the determination and quantification of steviol glycosides in *S. rebaudiana* samples [20, 21]. We analyzed seven different *S. rebaudiana* commercial extracts of different origins that are used in food and confectionery manufacturing and quantified the two main steviol glycosides present in stevia leaves, namely stevioside and rebaudioside A, plus rebaudioside B and the aglycone isosteviol. The data provided can help in selecting the most suitable commercial extract for use as a sweetener in food, beverage and confectionery manufacture.

Material and methods

Chemicals

The standard sugar rebaudioside A was purchased from Sigma-Aldrich (Italy), while rebaudioside B, isosteviol and stevioside were from Fluka (Italy). Warfarin sodium salt was purchased from Sigma-500 MAldrich (Italy) and used as an internal standard. Methanol, acetone and formic acid, all of >99% purity, were from Carlo Erba (Italy). The seven stevia raw samples were purchased from different European suppliers and are here numbered from 1 to 7.

Development and validation of the experimental protocol

The following pure standard steviol glycosides were used to develop the analytical method: stevioside, rebaudioside A, rebaudioside B and isosteviol. A 10 mg sample of each glycoside and 10 mg of warfarin were dissolved in 2 ml of methanol and diluted to a final concentration of 5 ng/ml in 0.1% formic acid/acetonitrile solution (1:1, vol/vol). Steviol glycosides were then infused into the ion source of a triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (API 3000, AB Sciex). The flow rate was set at 10 µl/min and the following parameters were used: nebulizer gas, 7; curtain gas: 12; collision gas: 4; ion spray voltage: 5500; temperature: ambient.

The ESI-MS/MS spectra for each glycoside and the internal standard were obtained using positive ionization and product ion scan mode (Fig. 1). For each steviol glycoside, we selected the two most sensitive fragmentation product ions. The first, defined as the quantifier, was selected for quantification of the glycoside and the second, the qualifier, was used together with the quantifier for its unambiguous qualitative identification. The selected precursor and product ions are reported in Table 1.

HPLC-MS/MS analysis

Steviol standards were first injected singly into the HPLC-MS/MS system to set up the chromatographic conditions. For HPLC analysis of the sugars, solutions containing the four steviol glycosides and the internal standard warfarin were

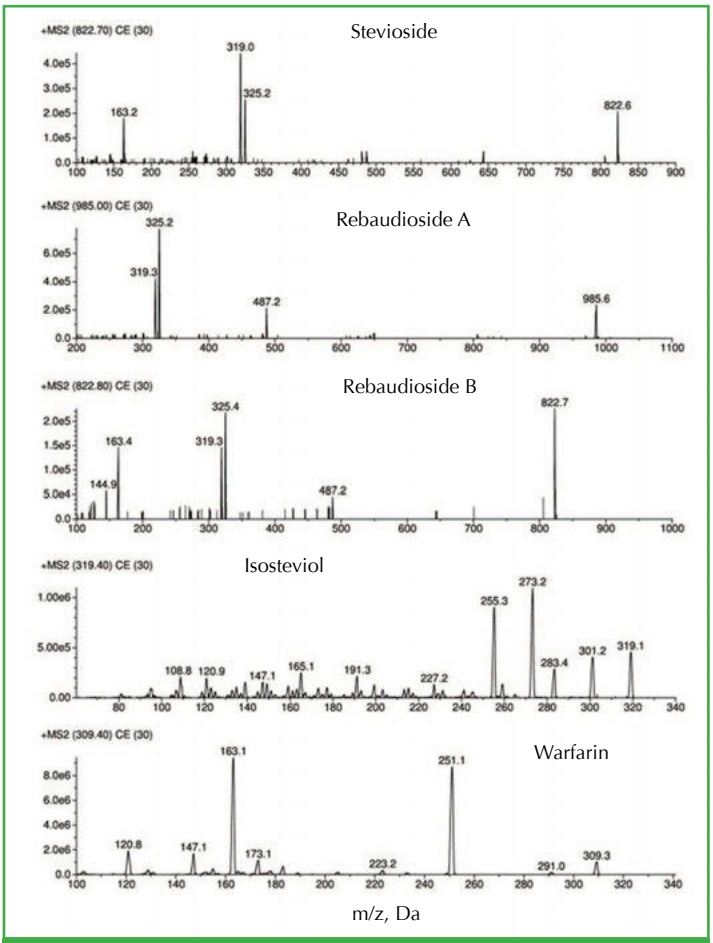

Figure 1 - ESI-MS/MS spectra of the standard steviol glycosides

Sugar	Precursor ion (m/z)	Product ion (quantifier) (m/z)	Product ion (qualifier) (m/z)
Stevioside	822.7	318.8	643.6
Rebaudioside A	985.6	325.4	487.0
Rebaudioside B	822.7	325.4	163.4
Isosteviol	318.9	273.4	255.4
Warfarin (internal standard)	309.4	163.2	251.1

Table 1 - Precursor ions and product ions selected for each steviol glycoside and for the internal standard

diluted to a final concentration of 5 ng/ml. Sugars were then separated by reverse phase chromatography (RPC) using a Series 200, Perkin Elmer HPLC system equipped with a X Select CSH C18 column (3.5 µm, 150×2.1 mm; Waters). The flow rate was set at 250 µl/min and the chromatographic conditions were as follows: Buffer A: 0.1% formic acid in H_2O; buffer B: acetonitrile.

Gradient: min 0, A 92% and B 8%; min 1, B to 80% in 5 min; min 6, B 80% for 2 min; min 8, B to 99% in 1 min; min 9, B 99% for 2 min; min 11, B to 8% in 1 min; min 12, B 8% for 8 min.

After separation, samples were introduced into the ESI source and chromatograms were obtained using a multiple reaction monitoring (MRM) acquisition mode with the transitions listed in Table 1. The chromatograms for the standard steviol glycosides are shown in Fig. 2.

Sensitivity and linearity of the method

A 10 mg sample of each steviol glycoside was dissolved in 2 ml of methanol and diluted with 0.1% formic acid/acetonitrile (92:8, vol/vol) solution. Stevioside, rebaudioside A and rebaudioside B were diluted in a concentration range of 0.1–5 ng/ml and 1–40 ng/ml solutions were prepared for isosteviol. The internal standard was dissolved at 5 mg/ml in methanol and diluted to 50 ng/ml with 0.1% formic acid/acetonitrile (92:8, vol/vol) solution. A 5 µl sample of warfarin solution was then added to 45 µl of each steviol glycoside to a final warfarin concentration of 5 ng/ml. Then 10 µl of each standard solution were analyzed as previously described and the signal-to-noise (S/N) ratio determined from the chromatograms for the analysis of each steviol glycoside at 10 pg/µl. The S/N ratio was calculated from the steviol specific signal and the baseline signal. The limit of detection (LOD) and the limit of quantification (LOQ) of the method were then calculated according to the following formulae: LOD=(10 pg/µl×3)/(S/N) and LOQ=(×10)/(S/N), where 3 and 10 are conventional values. The calibration curves were then plotted for each steviol glycoside and the linearity of the method was determined. These curves were used to calculate the concentrations of the glycosides in the raw materials.

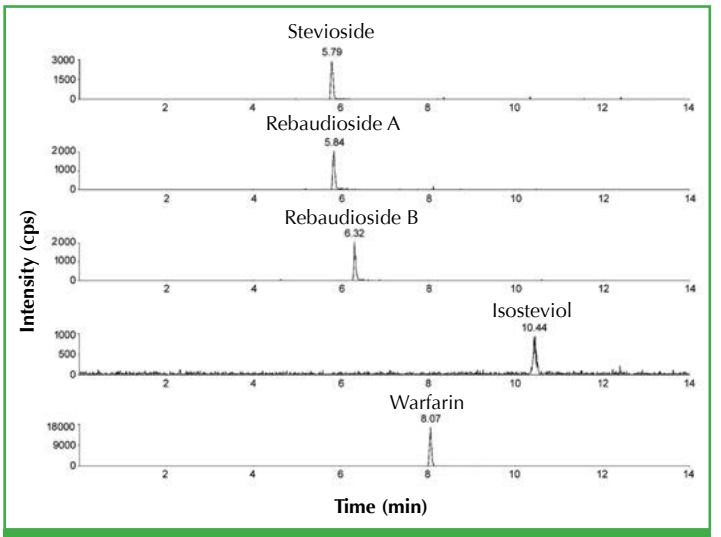

Figure 2 - Chromatograms showing the internal standard warfarin and the steviol glycosides. Each glycoside and isosteviol was injected singly at a concentration of 5 ng/ml

Analysis of stevia raw materials

A 10 mg sample of each stevia raw material was analyzed. Samples were dissolved in 1 ml of methanol and diluted with 0.1% formic acid/acetonitrile solution (92:8, vol/vol) at 20, 2 and 0.2 ng/ml. The internal standard warfarin was dissolved at 5 mg/ml in methanol and diluted to 50 ng/ml with 0.1% formic acid/acetonitrile (92:8, vol/vol) solution. A 5 µl aliquot of warfarin solution was then added to 45 µl of each stevia sample to a final concentration of 5 ng/ml. Then 10 µl of each solution were analyzed at least in triplicate, as previously described.

Results and Discussion

We used a very sensitive method for the qualitative and quantitative analysis of the steviol glycoside composition of stevia preparations routinely used in the food industry. This method is based on HPLC separation of the three steviol glycosides and isosteviol, coupled with ESI-MS-MS. The LOD and the LOQ for the steviol glycosides are reported in Table 2. The LOD and LOQ were low for stevioside, rebaudioside A and rebaudioside B, but higher values were obtained for isosteviol (Table 2). These results indicate that the method is more sensitive for the determination and quantification of stevioside, rebaudioside A and rebaudioside B than for isosteviol.

The calibration curves for the three steviol glycosides and for isosteviol are reported in Fig. 3. Linearity was maintained in the range of 0.1–5 ng/ml for stevioside (regression coefficient, R^2=0.9921), rebaudioside A (R^2=0.9938) and rebaudioside B (R^2=0.9936) and in the range of 1–40 ng/ml for isosteviol (R^2=0.9957).

This method was then applied to analyze steviol glycoside in the seven samples of stevia raw materials. Good chromatographic separation and MS-MS analysis were obtained for all seven samples (Fig. 4).

The quantification results, expressed as mg of compound/g of raw material, are reported in Table 3. Samples 2 and 7 consisted of around 100% steviol glycosides, with other samples consisting of 73–90% steviol glycosides (Table 3). Rebaudioside A was the most abundant component in all seven samples, ranging from 500 to 1000 mg/g of raw material (Table 3).

Steviol glycoside	S/N	LOD (pg/µl)	LOQ (pg/µl)
Stevioside	24.3	12.34	41.15
Rebaudioside A	26.5	1.13	3.77
Rebaudioside B	18.2	8.24	24.47
Isosteviol	14.7	408.16	1360.50

Table 2 - Signal-to-noise (S/N) ratio, limit of detection (LOD) and limit of quantification (LOQ) for the steviol glycosides analyzed

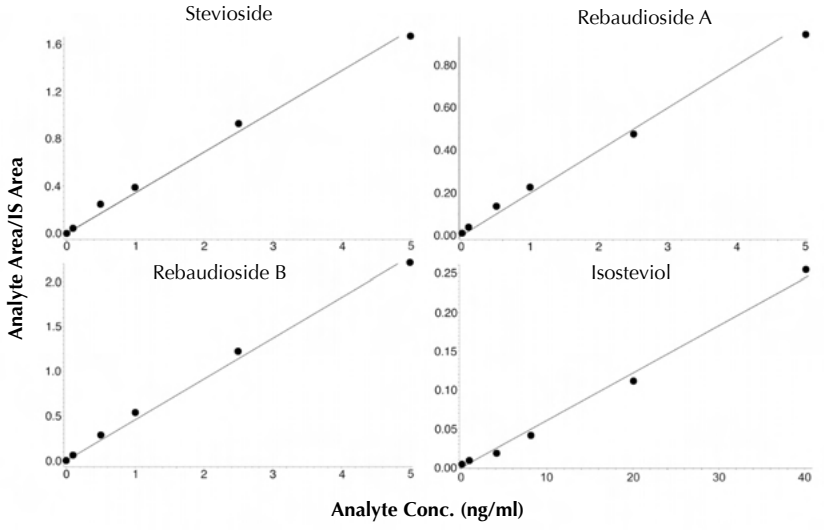

Figure 3 - Calibration curves obtained for rebaudioside A, rebaudioside B, stevioside and isosteviol. IS internal standard

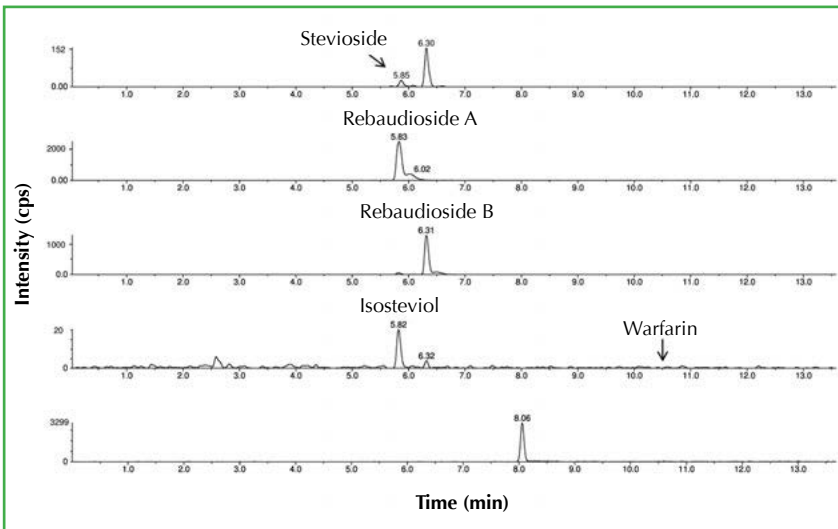

Figure 4 - Representative chromatograms obtained from analysis of sample 1, stevia raw material

Stevia sample	Stevioside (mg/g±SD)	Rebaudioside A (mg/g±SD)	Rebaudioside B (mg/g±SD)	Isosteviol (mg/g±SD)	% Of steviol glycosides in raw material
1	3.6±0.2	614.0±69.5	113.8±18.0	<LOD	73
2	3.5±0.1	1019.8±166.0	5.3±0.1	<LOD	100
3	2.7±0.3	701.0±14.1	130.8±10.7	<LOD	83
4	312.8±5.2	496.6±58.3	20.5±1.8	<LOD	83
5	84.7±4.2	728.2±75.0	85.3±7.3	<LOD	90
6	56.4±0.3	784.2±72.1	51.7±0.7	<LOD	89
7	386.0±36.9	598.0±64.3	39.4±5.0	<LOD	100

Steviol glycoside content is expressed as mg of compound/g of raw material.
<*LOD* below the limit of detection

Table 3 - SSteviol glycoside content in the stevia raw materials

Sample 2 was composed entirely of rebaudioside A. Rebaudioside B concentration ranged from 5 to 130 mg/g of raw material, while stevioside concentration ranged from 2.7 to 386 mg/g of raw material (Table 3). Isosteviol was under the LOD in all samples (Table 3).

One of the main concerns of the food, beverage and confectionary industry is to ensure that their products always taste the same. This is particularly difficult if they are using natural extracts of different origin, especially stevia extracts produced in various countries.

Different origins and extraction procedures may change the amount of ingredient present and therefore influence the overall taste of the product. One way to minimize this problem is to carry out sensorial tests using trained personnel, but this is still based on subjective judgment. We wanted to characterize the main concentrations of the components responsible for the taste of stevia extracts in a relatively short time. This approach aimed to provide information on the taste of the different extracts based on their qualitative and quantitative composition.

Conclusions

Our results from analysis of seven commercial stevia extracts indicate that the composition of each is quite different, confirming that the taste of each one may also be quite different. Careful chemical analysis can therefore ensure that in-house mixtures contain a constant proportion of the relevant stevia components. This is an important goal for industrial production in order to meet consumer expectations.

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Human and animal rights

This article does not contain any studies with human or animal subjects performed by the any of the authors.

Informed consent

This article does not contain any studies with human or animal subjects performed by the any of the authors.

Conflict of interest

The Department of Molecular Biochemistry and Pharmacology of the IRCCS- Istituto di Ricerche Farmacologiche "Mario Negri" has received scientific consultancy fees from Perfetti Van Melle SpA within the last 5 years.

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