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Analytical Methods

Fast methodology of analysing major steviol glycosides from *Stevia rebaudiana* leaves

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ABSTRACT

The aim of this work is to propose an HPLC method for analysing major steviol glycosides as well as to optimise the extraction and clarification conditions for obtaining these compounds. Toward this aim, standards of stevioside and rebaudioside A with purities ⩾99.0%, commercial samples from different companies and *Stevia rebaudiana* Bertoni leaves from Paraguay supplied by Insobol, S.L., were used.

The analytical method proposed is adequate in terms of selectivity, sensitivity and accuracy. Optimum extraction conditions and adequate clarification conditions have been set. Moreover, this methodology is safe and eco-friendly, as we use only water for extraction and do not use solid-phase extraction, which requires solvents that are banned in the food industry to condition the cartridge and elute the steviol glycosides. In addition, this methodology consumes little time as leaves are not ground and the filtration is faster, and the peak resolution is better as we used an HPLC method with gradient elution.

1. Introduction

In recent years, consumers are more and more worried about health, seeking natural and dietetic products. In this sense, the interest in *Stevia rebaudiana* Bertoni has increased considerably, as this plant has a high concentration (approximately 4–20%) of sweet diterpene glycosides in its dry-leaf matter (Geuns, 2003; Ghanta, Banerjee, Poddar, & Chattopadhyay, 2007). The high sweetness of the steviol glycosides makes them an attractive sugar substitute for food industries (Crammer & Ikan, 1986). Moreover, these glycosides are non-caloric sweeteners that reduce blood glucose and protecting the organism from diseases such as diabetes and obesity, among others (Geuns, 2003; Anton et al., 2010). Moreover, the steviol glycosides are related to other benefits, such as anti-hyperglycaemic, anti-hypertensive, anti-inflammatory, anti-tumour, antidiarrheal, diuretic and immunomodulatory effect (Chatsudthipong & Muanprasat, 2009). For all of these reasons, the steviol glycosides are known as the "sweeteners of the future" (Esmat, Azza, & Ferial, 2010; Brahmachari, Mandal, Rajeev, Mondal, & Brahmachari, 2011; Lemus-Mondaca, Vega-Galvez, Zura-Bravo, & Ah-Hen, 2012; Rao, Reddy, Ernala, Sridhar, & Ravikumar, 2012).

The main sweet components present in *S. rebaudiana* Bertoni are stevioside and rebaudioside A, representing 90 wt.% of all sweet glycosides in the leaves (Bergs, Burghoff, Joehnck, Martin, & Schembecker, 2012). Moreover, they have the most sweetness compared to sucrose (stevioside between 270 and 280 times more and rebaudioside A between 350 and 450). Probably for these reasons stevioside is the most studied glycoside (Montoro et al., 2013; Catharino & Santos, 2012) followed by rebaudioside A.

Other diterpene glycosides present in lower concentrations are steviolbioside, rebaudioside B, C, D, F, dulcoside A and rubusoside. The analytical determination of single diterpene glycosides by chromatography is a difficult task because of the chemical structures of the glycosides (Fig. 1) are similar (FAO, 2010), and because the aqueous extracts of Stevia leaf have many impurities such as proteins, resins, organic acids, pigments and sesquiterpene lactones, among others (Kovylaeva et al., 2007).

Many and varied extraction solvents and methods for steviol glycosides of Stevia leaves have been described in the literature: with chloroform and methanol (Kolb, Herrera, & Uliana, 2001), supercritical fluid extraction (Pól et al., 2007), by means of microwaves (Jaitak, Singh, & Kaul, 2009) and ultrasonics and enzymatic extraction (Jaitak et al., 2009; Liu, Li, & Tang, 2010; Puri, Sharma, & Tiwari, 2011; Puri, Sharma, Barrow, & Tiwari, 2012). In addition, there is abundant literature available with respect to the clarification and purification of these extracts (Zhang, Kumar, & Kutowy, 2000; Vanneste et al., 2011; Chhaya Sharma, Mondal, Majumdar, & De, 2012; Rao et al., 2012; Li, Chen, & Di, 2012), and several methods have also been reported for these extracts' analysis: Kovylaeva et al. (2007), Henderson and Berry (2009), Gardana, Scaglianti, and Simonetti (2010), Woelwer-Rieck, Lankes,

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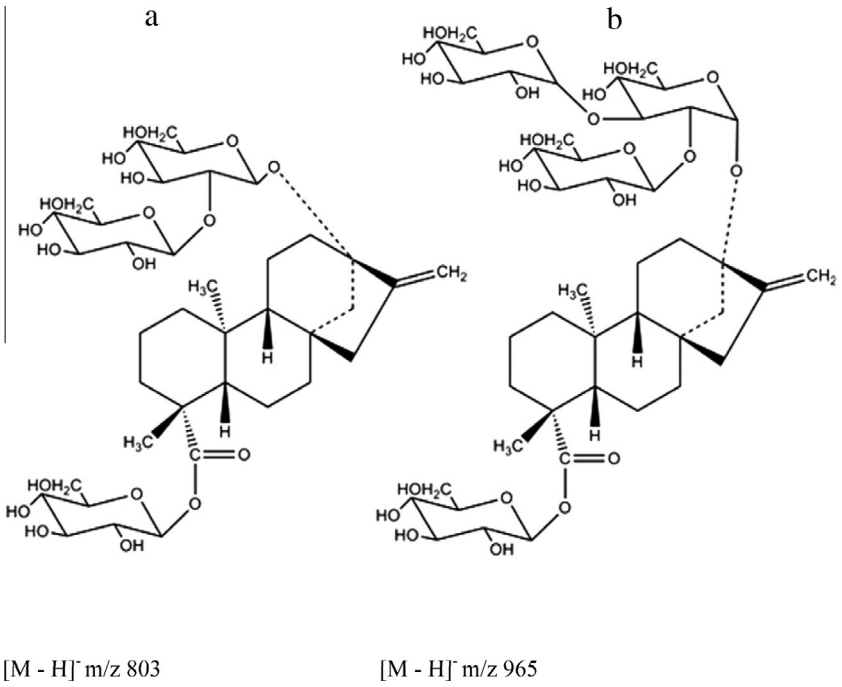

Fig. 1. Molecular structure of stevioside (a) and rebaudioside-A (b).

Wawrzun, and Wüst (2010), Bergs et al. (2012). However, for the application of the sweeteners as food additives, several restrictions and specifications must be taken into account (JECFA, 2007). For example, the use of solvents should be avoided as much as possible.

The aim of this work was to propose a method for the analysis of the major steviol glycosides in *S. rebaudiana* leaves. Towards this goal, the optimum conditions for the extraction of these glycosides are proposed; in addition extract clarification is accomplished by microfiltration and ultrafiltration (UF) and quantification by HPLC-DAD, avoiding where possible the use of dangerous solvents that are environmentally damaging.

2. Materials and methods

2.1. Samples

Different brands of commercial samples with several steviol glycosides concentrations, which were described on their labels, were used: Majota pill and powder (60% and 90% steviol glycosides), Masso 60% and 98% steviol glycosides, Azelis 95% and 98%, Cargill 98% and pure Stevia 99% steviol glycosides.

Dried leaves of *S. rebaudiana* Bertoni from Paraguay, supplied by Insobol, S.L., were used. The samples were dried at room temperature to a moisture level between 5% and 6% as determined with a halogen lamp moisture balance model XM-120T (Cobos, Barcelona, Spain) at 105 °C. When the moisture loss was less than 0.1% in 180 s, it was considered that the samples had reached a constant mass.

2.2. Reagents and standards

HPLC-grade acetonitrile was used from Panreac Química, SAU (Castellar del Vallés, Barcelona, España). Ultra-high-purity water was produced using a Milli-Q System from Millipore (Bedford, MA), and PVDF filters (13 mm, 0.45 μm) were also purchased from Millipore.

Stevioside and rebaudioside A with purities ⩾99.0% were purchased from Phytolab (Vestenbergsgreuth, Bavaria, Alemania).

The calibration solutions for the HPLC analysis of stevioside and rebaudioside A were prepared by diluting the analytes with acetonitrile/water (8:2 v/v).

2.3. HPLC conditions

The HPLC conditions were fixed with the standards cited above and diluted with acetonitrile/water (8:2 v/v) to 50 mg/L.

The liquid chromatographer used was an Agilent 1200 HPLC (Palo Alto, CA, USA). Two columns were tested: Develosil ODS-HG 140 Å 250 mm × 4.6 mm i.d., 5 μm and Luna HILIC 150 mm × 4.6 mm i.d., 5 μm Phenomenex (Le Pecq Cedex, France). Isocratic and gradient elution modes were tested. The eluents were water (A) and acetonitrile (B) in both systems, trying 80% B–20% A in the isocratic elution and several ramps in the gradient, as well as different elution times.

The oven was thermostated at 36 °C. The flow rate was 1 mL/min, and the sample injection volume was 20 μl. The DAD detector (Hewlett Packard, Waldbronn, Germany) was set at 210, 256, 330, 360 and 450 nm.

To confirm the chemical structure of the compounds, a Mass Spectrometer 6130 Quadrupole LC/MS, G1956 (SL) multimode electrospray and atmospheric pressure chemical ionisation (MM-ESI/APCI-MS) system was used, coupled to an Agilent Chem Station (version b.03.01) data-processing station. The parameters employed for MM-ESI–MS were dry gas, N_2, 10 mL/min; drying temperature, 350 °C; vaporizer temperature, 200 °C; nebulizer, 55 psi; capillary, 200 V (negative ionisation mode).

2.4. Method evaluation

Steviol glycosides were tentatively identified with the DAD detector by comparison with the corresponding UV–Vis spectra, on the basis of their molecular ion by electrospray ionisation mass spectrometry (MM-ESI–MS) (Fig. 1), and comparing with retention time of their pure standards in the chromatogram.