Jan M.C. GEUNS

Proceedings of the 2nd *Stevia* symposium
organised by EUSTAS 2008

Steviol glycosides: technical and pharmacological aspects

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CHAPTER 1

Preparation of steviol by soil bacteria

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ABSTRACT

Stevioside was hydrolysed to steviol using soil samples of a *Stevia* plantation in Paraguay. The best results were obtained with pasteurized soil samples that were incubated at 37 °C with a 0.2% stevioside solution. The responsible bacteria could be cultivated in and on a simple culture medium supplemented with stevioside. In the liquid cultures, full hydrolysis of stevioside to steviol was observed by TLC and HPLC analysis. All available evidence is in agreement with a two-step degradation by different micro-organisms *via* steviolbioside. Acidification of the culture

CHAPTER 2

Preparation of pure standards of steviol glycosides. Identification of steviol glycosides by LC-MS and NMR.

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ABSTRACT

Pure standards of the steviol glycosides were prepared from a commercial mixture of 70% pure steviol glycosides by silica gel, silica gel-NH_2 Flash column chromatography on columns of 300 mm length x 16 mm ID. Solvents used were ethyl acetate: ethanol: water (120:30:20) for silica gel columns and acetonitrile: water (70:30) for NH_2-columns. The eluates were checked by TLC. The combined fractions of standards were further purified by preparative HPLC on silica gel and on C_{18} columns. The obtained fractions were then crystallised from methanol. Identity of the different steviol glycosides was obtained by spectroscopic data: UV, NMR and MS (both in positive and negative ESI mode). Identity of the compounds in the *Stevia* leaves was confirmed by co-injections with pure standards, as well as by HPLC analysis on columns with totally different separation characteristics (NH_2 and C_{18} columns).

KEYWORDS: Steviol glycosides, stevioside, rebaudioside A, identification MS, NMR.

CHAPTER 3

Analysis of the main *Stevia rebaudiana* sweeteners and their aglycone Steviol by a validated LC-DAD-ESI-MS method

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ABSTRACT

Stevia rebaudiana (Stevia) leaves contain non-caloric sweeteners, mainly stevioside (SV) and rebaudioside A (Ra), widely used in many countries including Japan, Korea, China, Brazil and Paraguay. The aim of the present study was to develop and validate a simple and rapid LC-DAD-MS method to quantify SV and Ra and a LC-MS method to determine steviol (ST) in *Stevia rebaudiana* leaves and commercial extracts. Samples were extracted with 50% aqueous methanol for 2 h at 70° C and the diluted solutions purified by passing through an SPE cartridge. Steviol-glycosides were separated in isocratic mode by a 5 µm Luna-NH_2 (250 x 4.6 mm) using CH_3CN:H_2O (80:20, v/v) as mobile phase. Steviol was analysed by separation on a 3 µm Luna C_{18} column (150 x 2 mm) using CH_3CN:0.1% HCOOH (75:25, v/v) as mobile phase. Peak identity was established by co-chromatography, "on-line" UV spectra comparison and molecular ion evaluation. The least quantification limits (LLOQ) for SV, Ra and ST were 1.2, 2.5 µg/ml and 10 ng/ml, respectively. Assay validation demonstrated good performance in terms of accuracy (91-103 %), repeatability (6-11 %) and precision values (<9%). For stevioside and rebaudioside A, linearity was tested over a working range of 20-130 mg/g. The analyzed Stevia leaf extracts were characterized by the presence of different steviol-glycosides with the most abundant being stevioside (5-9%) and

CHAPTER 4

Analysis of Steviol glycosides: validation of the methods

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ABSTRACT

Pure steviol glycoside standards were used for validating the methods of analysis (Struyf *et al.*, 2008).

UV spectra of steviol glycosides and of steviol were measured in water, EtOH, 80% AcCN and 35% AcCN (5 mg/100 ml). The maximum absorption was around 196, 205, 199 and 197 nm in water, EtOH, 80% AcCN and 35% AcCN, respectively.

The calibration curves of steviol glycosides and of steviol were rectilinear between 15 and 1243 µM. The UV signal was monitored at 190 and 210 nm. It was shown that it is possible to make calibration curves with one single pure standard (eg. stevioside or rebaudioside A). The amounts of the other steviol glycosides present were calculated by use of conversion factors that compensated for the different molecular weights.

The detection limit (S/N = 5) of steviol glycosides was about 25 ng; that of steviol after derivatisation to its (7-methoxy-4-coumarinyl) methyl ester about 100 pg. Limits of quantification (S/N=10) are 50 ng and 200 pg for steviol glycosides and steviol respectively. The range of measurements is between 0.025 and 1000 µg/mL for steviol glycosides (or between about 15 and 1250 µM) and between 0.05 and 50 µg/mL for steviol as its fluorescent derivative (or between 15 nM and 160 µM).