Jan M.C. GEUNS

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Table of Contents

CHAPTER 1

The steviol glycosides of *Stevia rebaudiana*: chemical diversity, biosynthesis and metabolism

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ABSTRACT

Steviol glycosides are a group of highly sweet diterpene glycosides discovered in only a few plant species, most notably the Paraguayan shrub *Stevia rebaudiana* Bertoni (Bertoni). During the past few decades, the nutritional and pharmacological benefits of these secondary metabolites have become increasingly apparent. While these properties are now widely recognised many aspects related to their *in vivo* biochemistry and metabolism and their relationship to the overall plant physiology of *S. rebaudiana* are not yet understood. The current review intends to thoroughly discuss the available knowledge on these issues.

1. Introduction

In recent years, the development of natural sweeteners has been moving up a gear in order to provide alternatives for saccharose which are preferably non-calorific, non-cariogenic and generally healthy. The need for alternative sweeteners is expected to increase further, as metabolic disorders such as type-II diabetes and obesity are becoming ever more

CHAPTER 2

Molecular Basis of Steviol Glycoside Biosynthesis in *Stevia rebaudiana* (Bertoni), a Source of Non-calorific Sweetener

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ABSTRACT

Leaves of Stevia [*Stevia rebaudiana* (Bertoni)] are a rich source of steviol glycosides (SGs). Reflecting the importance of Sgs, an enormous literature exists on various related issues. However, the molecular basis of SGs biosynthesis needed attention. To understand the molecular basis of SGs biosynthesis, the cloning of genes of the pathway and their regulatory sequences occupies a central role. The present work was an attempt to understand the molecular basis of SGs biosynthesis in Stevia. Previously, a total of eight full-length genes were cloned from Stevia and the expression of fifteen genes was studied in relation to different organs including different leaf positions and in response to various phytohormones. Data identified *SrDXR*, *SrKO* and *SrNCYPR* as possible regulatory genes in the SGs biosynthesis pathway. Since information on promoters of genes of SGs biosynthesis was lacking, upstream regions of seven selected genes, namely, *SrMCT*, S*rCMK*, *SrHDS*, *SrHDR*, *SrIDI*, *SrGGDPS* and *SrNCYPR* were cloned and analysed. Analysis of regulatory elements in the promoters suggested a requirement for light and a hormone responsive regulation.

CHAPTER 3

Effect of Genotype and Environment on Steviol Glycosides Content and Composition in Stevia at Flower Budding Stage

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ABSTRACT

There is a growing interest in steviol glycosides (SVglys) since their approval by the European Union in December 2011. Numerous SVglys have been identified in stevia (*Stevia rebaudiana* Bertoni) leaves. The major ones are stevioside (ST), rebaudioside A (RA), rebaudioside C (RC) and dulcoside A (DA). Owing to its more pleasant sensory characteristics, more attention is given to RA. This component is one of the most glycosylated SVglys found at the end of the biosynthetic pathway of SVglys. However, this pathway is not completely elucidated. The objective of the present work was to evaluate the variability of SVglys content and composition in *Stevia rebaudiana* leaves among a range of clones. Agronomic practices and environment effects on SVglys content were also studied on 7 clones. This study showed good breeding potentialities for improvement of SVglys composition and total content. SVglys composition appeared less influenced by environment than SVglys total content. Significant correlations have been observed between the different SVglys, suggesting biosynthetic relationships between RA and RC.

KEYWORDS

Steviol glycosides, Genotype effect

CHAPTER 5

Sustainable Production Chain of Stevia in Italy: Agronomy, Phytochemical Assessment and Downstream Processing

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ABSTRACT

Objective: Since in Europe an increasing demand has been observed for stevia extracts to use in human diet or as an industrial primary product, large efforts are necessary to optimize stevia cultivation in terms of competitiveness and quality in order to tackle the existing bottlenecks and address the needs of the bio-industry. To date, the main stevia-producing areas are China, especially north China, and South Asia. In Italy, there is currently no large-scale growing of stevia or industrial plant for steviol glycoside extraction. Consequently, stevia is still an experimental crop and the agronomy of this alternative species should be further improved to achieve great steviol glycoside yield and quality level. For this reason, modern and site-specific techniques for a sustainable agricultural production of raw material should be identified in order to fulfill the market requirements for safe and high quality products. Since the early '90ties the Department of Agriculture, Food and Environment, of the University of Pisa started to study Stevia with the aim to define all the steps of the production chain through: i) the identification of optimal cultivation strategies (propagation techniques, genotype screening, timing of harvest, fertilization) to define a modern agronomical blueprint that could allow farmers to cultivate this crop in a sustainable and/or organic cropping system; ii) the optimisation of laboratory extraction procedures using appropriate and up-to-date technologies; iii) the valorisation of all the components present in the stevia leaf extracts, in particular the protein content and antioxidant compounds (polyphenols and flavonoids).

Materials and Methods: A series of field and laboratory trials have been done in order to assess the effect of genotype, cultural practices (nitrogen fertilization, harvest time and crop age) and propagation methods (micropropagation, stem cuttings and seed propagation) on leaf production, steviol glycoside content,

CHAPTER 6

Effects of Agricultural Techniques on the Quantitative and Qualitative Characteristics of Stevia rebaudiana Bertoni, under Mediterranean Conditions (Karditsa, Thessaly, Greece)

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ABSTRACT

Worldwide, Stevia cultivation is increasing steadily, with thousands of hectares being exploited with this natural sweetener plant. The present study represents the first large-scale field experiment of the performance of *Stevia rebaudiana* (Bertoni) in Greece, allowing the documentation of a wealth of information regarding the response of this species in a Mediterranean environment. Our experiments were conducted during 2008 - 2009, in a slightly alkaline soil of pH 7.6, using a basic 3-3-6 fertilisation (N-P-K). We used the Criolla variety and the plants were transplanted at the end of May. We focused particularly on the effect of different planting distances on: the maximum height of plant, the stem fresh weight, the leaf fresh weight, the root fresh weight, the stem dry weight and leaf dry weight. We used six different spacing systems (60 x 20, 60 x 40, 75 x 20, 75 x 40, 90 x 40 and 90 x 20 cm), for comparison.

Our preliminary results indicate a better plant performance with the 75 x 40 cm spacing system. In the 60 x 20 cm, spacing system of the largest number of plants compared to the remaining spacing systems, resulted in the greatest amount of harvested material, measured in kg dry leaf weight per hectare. The qualitative analysis of the concentration of steviol glycosides (stevioside and rebaudioside-A) revealed that the spacing system 75 x 40 cm also provided the largest concentration of glycosides in plant leaves. By contrast, the largest concentration of total glycoside percentage alone was found in the 60 x 20 cm spacing system. After harvesting, the plants were dried by an innovative method we devised, using

CHAPTER 7

Effect of Soil Moisture Variation and Nitrogen Fertilisation on *Stevia rebaudiana* Bertoni Glycosides Content and Yield

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ABSTRACT

Objective: Knowledge about the response of stevia to agronomic inputs is necessary in order to define a sustainable production system for this new crop. Although there are a few research studies about stevia, more scientific information on Stevia's requirements and adaptation to climate change is needed for agronomists. Water and fertilisation are the primary inputs in agriculture. So, to study the drought stress and nitrogen fertiliser effects on stevia steviol glycosides content and production, in 2012 two greenhouse experiments were done based on a completely randomised design with three replications.

Materials and Methods: Four week old micropropagated plants were transplanted in pots filled with a clay-loam soil. Greenhouse temperature, relative humidity and CO_2 were 25/22 °C, 60/40 % and 500/600 ppm, respectively. In the drought experiment, irrigation scheduling was applied every 3, 6, 9 and 12 d, when the soil moisture content in weight was 19, 15, 11 and 9 % respectively (field capacity 20.2 %; wilting point 10.5 %; bulk density 1.4 $g.cm^{-3}$). Five nitrogen amounts (0, 30, 60, 90, 120 and 150 kg/ha of nitrogen from urea) were applied in the nitrogen fertiliser experiments. Plants were harvested 64 d after transplanting and the morphological and productive traits were recorded. Steviol glycosides content was determined on dry leaves based on FDA (2009) method with some modifications. The data from the two different experiments were statistically analysed by One Way ANOVA and means were separated on the basis of Least Significance Difference ($P \leq 0.05$).

CHAPTER 8

Analytical Methods

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ABSTRACT

Stevia rebaudiana accumulates more than 35 different steviol glycosides in varying concentrations, depending on genotype and cultivation conditions. The best known steviol glycosides are stevioside and rebaudioside A, which are the main glycosides in the plant. A short survey about the different steviol glycosides will be given.

The analysis of steviol glycosides starts with the sample cleanup followed by a chromatographic separation, mainly by HPLC. Steviol glycosides with a purity of more than 95 % as approved since December 2011 need no further cleanup prior to quantification. The isolation of steviol glycosides from leaves or complex food matrices is more demanding and requires extraction and cleanup steps. Various techniques will be presented and discussed.

For the separation and detection of steviol glycosides high performance liquid chromatography (HPLC) is the method of choice. Different columns and mobile phases are in use. The advantages and disadvantages of these columns will be discussed and new types of columns will be presented.

The most employed detector is the UV-detector operating at a wavelength of 200 or 210 nm, which is a rather non-selective wavelength, but which is nevertheless indispensable for the weakly UV-absorbing steviol glycosides. UV-detection is going to be replaced by mass spectrometric detectors which are much more sensitive and selective. In the last ten years many methods have been published using mass spectrometry. Some of these methods will be presented and the different techniques as well as their sensitivity will be described and discussed.

CHAPTER 9

Testing of Steviol Glycosides: Influence of Different Commercial Standards on Purity Results

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ABSTRACT

In Europe, the purity of steviol glycosides has to be at least 95 % on a dry matter basis (Regulation 1131/2011 EU). Several products on the European market show important differences in purity after HPLC official method analysis depending on the laboratory, the parameters of the method employed and on the standard used (origin and type). There are several possible errors that could lead to such differences; among them, the quality of the standard used is the parameter we have analysed in this study. In order to show the impact of the standards, we have compared different commercially available standards from four suppliers and analysed identical samples with these standards to demonstrate that the choice and the way to prepare and to use the standards are critical issues for reliable results.

KEYWORDS

Steviol Glycosides purity, analytical methods, commercial standards, HPLC

CHAPTER 10

Analysis of Steviol Glycosides in *Stevia* Plant Material and Dietary Supplements Containing *Stevia* by Liquid Chromatography with Ultraviolet Detection

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ABSTRACT

With wider commercial use of *S. rebaudiana* sweeteners, the positive scientific opinion of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the regulatory approval for the use of steviol glycosides in foods and beverages by the European Food Safety Authority (EFSA), numerous sample preparation methods and analytical methods for the analysis of steviol glycosides have been published. Most analytical methods for the analysis of steviol glycosides have been carried out by using reversed phase columns or amino columns followed by high performance liquid chromatography with ultraviolet detection.

In this work, the separation of steviol glycosides by new analytical columns such as hydrophilic liquid interaction columns and mixed mode columns and their applicability for routine analysis will be discussed. Different approaches for the analysis of steviol glycosides in *Stevia rebaudiana* plant material, commercially available *Stevia* sweeteners and beverages containing stevioside and/or rebaudioside A will be shown.

CHAPTER 11

Development and Validation of a Fast HPLC-DAD-ELSD Gradient Method for Simultaneous Determination of Steviol Glycosides and Other Sweeteners

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ABSTRACT

The use of steviol glycosides as sweetener on their own is limited due to their liquorice and bitter after-taste as well as sweetness linger (Carakostas et al. 2012). Blending with non-caloric but synthetic sweeteners to mask this off taste is possible (Prakash et al. 2007). Few products are already commercially available, e.g. beverages and candies.

In this paper, we present the development of a HPLC-method for simultaneous detection and quantification of the ten steviol glycosides, specified by EU, in addition to the synthetic sweeteners acesulfame K, saccharin, aspartame, cyclamate and neohesperidine dihydrochalcone.

Due to the chemical and physical diversities of the analytes, a gradient HPLC protocol has to be developed to achieve a separation on a RP-18 endcapped phase. Cyclamate which has hardly a UV activity could not be detected by diode-array-detection (DAD). Thus an evaporative light-scattering detector (ELSD) was connected with the HPLC-DAD-system as it was possible to

CHAPTER 12

Curriculum Vitae: Nature's logistic legacy

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Abstract

The integration of natural ligands in membrane processes was explored with the eye to increasing the often moderate selectivity for the separation of different carbohydrate substrates. Sugar-selective bacterial porins could be used to manufacture the first chemical free membrane adsorber. Ultimately, bacterial strains could be selected, through multiple evolution cycles, that express porins tailored to recognize specific molecules. Although bacterial porins exhibit interesting sugar-selectivities, lectins, are definitely better suited for high resolution carbohydrate purification. Lectins could be used to effectively separate different sugar isomers in one bind and elution step. The potential of Concanavalin A for the purification of the natural sweetener stevioside was explored. A discrimination factor in dissociation constant of

CHAPTER 13

Pressurized Liquid Extraction Method For *Stevia Rebaudiana* Bertoni Screening

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ABSTRACT

The aim of this study was to establish a quick, reliable and reproducible extraction method allowing the screening of *Stevia rebaudiana* Bertoni genotypes for their content in steviol glycosides, at laboratory scale.

The pressurised liquid extraction method was chosen for this purpose. A response surface methodology with a three-factors Doehlert's type uniform network was applied to model and optimise the steviol glycosides extraction. After a validation of the model with a statistical analysis, first conditions were set. Then an optimization was done to reach a 100 % extraction yield in 13 minutes and 30 seconds.

KEYWORDS

Accelerated Solvent Extraction, Screening, Response Surface Methodology

CHAPTER 14

Microbial Degradation of Steviol Derived from Steviol Glycosides

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ABSTRACT

Bacterial consortia that hydrolysed steviol glycosides to steviol were derived from both Paraguayan soil samples of a stevia plantation and from samples of Belgian soils that never had contact with stevia or steviol glycosides. This activity was not influenced by heating (20 min 80 °C) or boiling (10 min 100 °C) the soil samples. The type of steviol glycosides that were hydrolysed, as well as the hydrolytic pathway of the hydrolysis, was highly influenced by the conditions of the incubation. Prolonged incubation with one of the consortia yielded two new compounds designated as Monicanone and dihydromonicanone. Full chemical characterisation showed that these compounds were formed from steviol by removing the A-ring.

KEYWORDS

Soil bacteria, steviol, β-glucosidase, Monicanone, Dihydromonicanone

INTRODUCTION

Steviol glycosides (**SVGlys**) are extracted from *Stevia rebaudiana* Bertoni and have an exciting potential for use as an innovative solution for calorie and sugar reduction. The human digestive system is unable to hydrolyse the β-glycosidic (ester) bonds that link the sugar moieties to the steviol (**SV**) scaffold and this explains why these compounds are calorie-free. However, bacteria in the colon do hydrolyse these bonds, thus setting steviol free for further metabolic processing (Geuns, Buyse, Vankeirsbilck, & Temme, 2007). Part of the steviol is taken up and is glucuronidated; in this way, steviol glucuronide is the only metabolite of SVGlys found in the human serum and so it is considered to be the active component responsible for some beneficial effects including anti-hypertensive, anti-inflammatory and anticancer effects (Chatsudthipong & Muanprasat, 2009). Basic research into the role of steviol glucuronide in the human body depends on its

CHAPTER 15

Best Practice Post-Market Surveillance for a Stevia-Based Sweetener

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Objective: The objective of this project was to establish and promote a "best practice" US approach to monitoring the safety of a steviol-glycosides-based, non-nutritive, sweetener intended for global launch into the direct-to-consumer market.

Materials and Methods: In 2008, Cargill Incorporated (Cargill) launched Truvia® brand sweetener containing highly purified steviol glycosides. Cargill partnered with the SafetyCall International Poison Center (SCI), an academically affiliated, triple board licensed healthcare firm to establish a 24/7 SCI medical call centre support line for any US consumers alleging product related adverse effects. An enhanced, product-specific questionnaire was also designed to aid in incident investigation.

Results: Four years of product-specific incident data have been collected allowing for normalisation of incidence rates compared to sales and distribution; comparison of normalised relative incidence rates against standard factors for fault analysis; investigation of unique safety hypothesis related to steviol glycosides and adverse events; and benchmark analysis against blinded data within, across, and outside the product class. The overall incidence rate for any report involving one or more

CHAPTER 16

A Chemical View Of The Stevia Market

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ABSTRACT

Objective: The quality of stevia extracts, food containing steviol glycosides (including table top sweeteners) and stevia leaves is summarised.

Materials and Methods: Institut Kurz is a commercial laboratory in Germany that established the analysis of stevia products long before steviol glycosides were allowed in the European Union (EU) (Zimmermann 2011, Zimmermann et al. 2012). The analysis includes steviol glycosides and steviol as well as more generic analyses such as solvent residues, heavy metals, microbiological contamination and more, including all the parameters required by the EUSTAS label. In the last two years Institut Kurz analyzed more than 400 stevia samples from various European and foreign clients.

Results: Although it is not known, if the samples analysed are representative, the large number of samples gives a good impression on what is on the market and which parameters of the EU or EUSTAS requirements are more often not fulfilled.

Conclusion and Broader Impacts: Some quality parameters are usually satisfactory, but others are often outside the required specifications. The content of steviol is not mentioned in the EU regulation, but in the EUSTAS criteria its "absence" is required, since steviol is discussed as being toxic. From an analytical point of view the term "absence" depends on the sensitivity of the method used and should therefore be substituted by a concrete figure.

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