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Stevia: Six months beyond authorization

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CHAPTER 1

Influence of photoperiodism on the spatio-temporal accumulation of steviol glycosides in *Stevia rebaudiana* (Bertoni)

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ABSTRACT

The effect of photoperiodism on the accumulation dynamics of steviol glycosides (SVglys), glucose and sucrose was investigated in *Stevia rebaudiana*. Topped plants were grown under a 16 h or 8 h photoperiod and left to develop new shoots. At different stages during ontogeny, leaves, stem material, lateral shoots and roots were collected, as well as the generative organs. All samples were analysed for SVgly and carbohydrate content. Long-day (LD) conditions prolonged vegetative growth, thereby significantly increasing leaf biomass and SVgly amounts. During LDs, absolute SVgly amounts per shoot decreased during flower bud formation, while under short-days (SDs) this occurred after flower opening. Spatially, these decreases were restricted to mature leaves in LD conditions or young leaves in SD conditions. When lateral shoots were included in plants under LDs, leaf yield, absolute and relative SVgly amounts significantly increased, indicating the possibility of a post-flowering harvest under LDs with relatively greater yields. The commercially interesting ratio of the level of rebaudioside A (Reb A) to stevioside (ST) was influenced by ontogenetic stage and daylength, with higher ratios during vegetative stages of plants under SDs. The larger fluctuations in minor SVglys were equally affected by daylength, with more fluctuations seen under SDs. At the organ level, the largest variations were measured in the reproductive organs,

CHAPTER 2

Effect of red LED light on steviol glycoside accumulation and transcriptional analysis of the genes involved

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ABSTRACT

The effect of red LED light on the biosynthesis of steviol glycosides (SVglys) was investigated by a transcriptional analysis of the genes involved. Plants were grown under short-days (8 h light; SD), long-days (16 h; LD) or SDs with a red LED light treatment during the night (SD + LED). After 76 d of incubation, SD groups were fully flowering, while the other groups were still vegetative. Transcription of some of the biosynthetic genes decreased after the onset of generative development, particularly *ent-KS* and *UGT85C2*, whereas the other genes remained fairly constant. Comparing to the upper leaves of young seedlings at t_0, little or no significant changes were seen in the LD and SD + LED groups during ontogeny, or between both groups. Likewise, little or no significant spatial differences were observed across the stem. It is likely that gene transcription is mainly influenced by ontogeny, which itself is affected by photoperiod. The influence of red LED light on gene transcription is an indirect effect of the prolongation of vegetative growth under these conditions. Treatment

CHAPTER 3

Stevia in the field – Challenges

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ABSTRACT

There are innumerable factors that influence the results of growing stevia in bigger fields. The size of the area of cultivation, the environment, planting, maintenance of the field, harvesting and drying – to name a few – are variables that will define the success of stevia production.
The interaction between these field conditions and how the challenges resulting from the day-to-day conditions are handled will determine whether or not business will be prosperous.

KEYWORDS
Stevia cultivation, field conditions, interrelated variables, challenges.

Challenges from a South American point of view

I. Area of cultivation

The so-called traditional stevia cultivation area used to be below 1 hectare (10,000 m^2). The small farmers in the original stevia region in Paraguay cultivated the plant in their backyard or with their vegetables in a garden plot. The cultivation in Asia was originally organized the same way: small farmers received the seedlings, cultivated them on small areas (less than 1 ha) and delivered the dry leaves to the collection centres.
If stevia is cultivated on small areas, the plant has to be cared for the same way as one would do with vegetables on the garden plot. The difference is that stevia is a perennial plant so it will remain on the same spot for 5 or more years. The challenge resides in maintaining the stevia area clean of weed and diseases over the span of 5 years.

CHAPTER 5

First steps towards an *in vitro* and cryopreserved Stevia collection

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ABSTRACT

The first steps were taken in order to establish an *in vitro* collection for different Stevia accessions. Pot plants were obtained from different sources, apical as well as axillary shoots were excised and a sterilisation procedure was defined. Next, an optimal shoot culture medium was developed. For this purpose, 4 different *in vitro* culture media were compared: P5, MS, "Stevia" and WPM. Shoot and roots were measured and the simplest medium MS, was selected for our purposes.

Cold storage was tested at 6 and 15 °C and compared with normal culture conditions at 25 °C. We observed that storage of stevia in *in vitro* cultures for up to one year at 15 °C is possible without subculture.

Additionally, a first preliminary cryopreservation experiment was executed using droplet vitrification. For this, apical meristems of *Stevia ovata* and one Stevia cultivar were subjected to droplet vitrification. Results are expected soon.

CHAPTER 6

Preparative separation of rebaudioside A from Stevia leaves using inverted chromatographic process design[1] - An overview

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[1] Extension of B. Burghoff and D. Bergs: Purification of rebaudioside A from *Stevia rebaudiana* Bertoni, Proceedings of 5th Stevia symposium, organised by EUSTAS 2011.

CHAPTER 8

Round Robin Test for the Analysis of Steviol Glycosides launched by the International Stevia Council

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ABSTRACT

In May 2011, the International Stevia Council launched its Proficiency Testing Program (PTP). The PTP ensures that consistent analytical methods and reference standards are used throughout the industry in measuring steviol glycoside content and is designed to help companies determine the quality of their Stevia products.

16 companies of which 5 were not members of the ISC, participated in the first year programme including stevia extract producers, food and drink companies, universities and test-laboratories that analyse stevia extracts.

The PTP included 4 rounds with 3 samples each. All samples were highly purified Stevia extracts, i.e., with a total percentage of steviol glycosides over 95 %. Some samples were sent out twice (of which the participants were not aware). The results were satisfying, when samples with a large Rebaudioside

CHAPTER 9

EUSTAS Protocol and Round-Robin testing of steviol glycosides by an internal standard method

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ABSTRACT

The internal standard (IS) method is the best method for the analysis of samples, as it is independent of errors in injection volume, changes in sample volumes, changes in sensitivity of the detector, etc. Moreover, use of an internal standard allows for the correction of losses due to sample clean-up of complex samples (e.g., after extraction of foods). An ideal IS is a compound with properties very similar to, and that behaves in a similar way to the compounds to be analysed. Ideally, only in the last step of analysis (HPLC), should the IS be well separated from the compounds of the mixture to be analysed. We synthesized 19-*O*-β-D-galactopyranosyl-13-*O*-β-D-glucopyranosyl-steviol as an IS.

The results show that the analyses of SVglys using an IS are very much simplified. The possible errors are much reduced. The inter-laboratory RSD for the analysis of all the SVglys present was about 1.75 %, which is much better than can be obtained by an external standard method. This value might still decrease after improvement of peak resolution and peak integration techniques in some laboratories. Our method made it possible to inject 5 × more of the same sample vials, resulting in a more accurate measurement of the small peaks, enhancing overall accuracy. Besides the analysis of SVglys, also the amount of SVeqs is given, expressed on a dry and wet wt. basis. The IS method is likely to become the method of choice for the whole Stevia industry.

KEYWORDS: Testing steviol glycosides; internal standard method; HPLC.

Abbreviations: Steviol glycosides, the sweeteners of Stevia, are sometimes abbreviated as follows. Using SV for steviol, allows the following abbreviations to be used: SVgly(s) for steviol glycosides, SVeq for steviol equivalents, SVglu for steviol glucuronide, SM: steviol monoside, SVE: steviol-19-ester, ST: stevioside,

CHAPTER 10

Validation of an internal standard method for direct measurement of steviol equivalents in foods

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ABSTRACT

An internal standard RP-HPLC-fluorescence method was developed and validated to quantify directly steviol equivalents in commercial steviol glycoside mixtures and different food matrices. This is based on a post-derivative quantification of isosteviol, formed by acid hydrolysis of extracted steviol glycosides, using dihydroisosteviol as internal standard. The calibration curves were linear over the range 0.013 mM – 1.61 mM of steviol equivalents, with a quantification limit of 164 pmol. The percentage relative standard deviations of intra-day precision varied between 0.4 % and 3.9 %. The inter-day precision varied between 0.4 % and 5 %, for high and mid concentrations and between 3 % and 8 % for small concentrations. Accuracy, expressed as recovery of spiked amounts of steviol glycosides, varied between 99 % – 115 % for analysis of commercial steviol glycosides mixtures and different food matrices.

KEYWORDS: steviol glycosides; steviol equivalents; isosteviol; acid hydrolysis; dihydroisosteviol; derivatization.

Introduction

Steviol glycosides (SVglys), the sweet diterpene glycosides found in *Stevia rebaudiana* Bertoni, have been used as intense sweeteners for many years. In several countries they are allowed in general food (China, Russia) or as a food

CHAPTER 11

Radical scavenging activity of steviol glycosides, steviol glucuronide and crude Stevia extracts.

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ABSTRACT

A radical scavenging activity, expressed as the inhibitory concentration (in mM) giving 50 % reduction of radicals (IC_{50}), of steviol glycosides, steviol glucuronide and crude Stevia extracts has been demonstrated *in vitro*. Quercetine and ascorbic acid were used as a positive control. The activity on hydroxyl radical ($^{\bullet}OH$) scavenging was measured by the decrease in the fluorescence of hydroxyterephthalate that is formed from terephthalate in the presence of hydroxyl radicals. The positive controls ascorbic acid and quercetine had an IC_{50} of 1.154 and 0.115, respectively. Stevioside, rebaudioside A and rubusoside had about the same scavenging activity (IC_{50} between 0.196 and 0.278) and were much better than glucose or sucrose (IC_{50} = 1.119 – 1.562). Steviol glucuronide, the excretion product in urine, also had strong ROS ($^{\bullet}OH$) scavenging activity (IC_{50} = 0.206). The crude water extracts of Stevia leaves and stems were very potent radical scavengers. PVPP could remove part of the scavenging effect of *S. rebaudiana* extracts, whereas active charcoal removed about 50 % of the scavenging activity.

Superoxide radicals ($O_2^{\bullet-}$) were most efficiently scavenged by the positive control ascorbic acid (IC_{50} = 0.159 mM), followed by steviol glucuronide (IC_{50} = 0.211) and quercetine (IC_{50} = 0.320). Steviol glycosides had only a moderate superoxide scavenging activity (IC_{50} ≈ 1.5 - 2.5 mM). All crude plant extracts (leaves and stems) had an excellent superoxide scavenging activity. PVPP could remove part of the scavenging effect of *S. rebaudiana* extracts, whereas active charcoal removed about 50 % of the scavenging activity.

Quercetine (IC_{50} = 0.9 mM) followed by ascorbic acid (IC_{50} = 11.3 mM) were active in limiting the amount of TBA reactive material. The other compounds were less active. However, crude plant extracts mainly of *S. rebaudiana* strongly reduced the radicals. Their scavenging activity was decreased by treatment with PVPP and active charcoal.

CHAPTER 12

Isolating the Risk Factors of Pancreatic Cancer

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Pancreatic Cancer

- 4th leading cancer related death
- 95% mortality in 12 months
- Late diagnosis after tumor is of respectable size
- No good bio-marker
- Hard do identify contribution of each risk factor through human epidemiological studies (12.1 per 100,000)
- Highly diverse risk factors include:

  -age **-diet** **-**race -**diabetes**

  -smoking -chronic pancreatitis -**obesity**

  -lack of physical activity

  -Genetic mutations (p16, PRSS1, MLH1, MSH2, STK1)