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BRIEF COMMUNICATION

Steviol glycoside content in different organs of *Stevia rebaudiana* and its dynamics during ontogeny

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Abstract

The contents of three major steviol glycosides (SGs) (stevioside and rebaudiosides A and C) in vegetative and generative organs during ontogeny of *Stevia rebaudiana* Bertoni were analysed with HPLC. Plant organs contained different amounts of the SGs, which declined in the following order: leaves, flowers, stems, seeds, roots. The highest content of the SGs was found in upper young actively growing shoot sections, whereas lower senescent shoot sections exhibited the lowest amount of such compounds. During ontogeny a gradual increase in the SG content was observed in both mature stevia leaves and stems, and this process lasted up to the budding phase and the onset of flowering.

Additional key words: HPLC, rebaudiosides A and C, stevia, stevioside.

Diterpenoids are a relatively non-numerous and little studied group of the secondary metabolism compounds. Some diterpenoids have unique properties and, therefore, their investigation is of special interest in view of their both fundamental and applied importance. For example, leaves of stevia contain a number of high sweet steviol glycosides (SGs), that are low-caloric, non-toxic and non-mutagenic (Lyakhovkin *et al.* 1993, Matsui *et al.* 1996). Such compounds are highly perspective for using them as sugar alternatives for patients suffering from the diseases related to disturbance in sugar metabolism (Kinghorn and Soejarto 1986, Bondarev *et al.* 1997). Major SGs are stevioside and rebaudiosides A and C (Tamura *et al.* 1984, Bondarev *et al.* 2001). At present, however, little is known about the distribution of SGs in stevia plants.

There are enough reports related to the composition and content of the SGs in stevia leaves (Kohda *et al.* 1976, Makapugay *et al.* 1984, Tamura *et al.* 1984, Nakamura and Tamura 1985, Lyakhovkin *et al.* 1993). In other organs of stevia (flowers, stems and roots) such an information is extremely limited (Zaidan *et al.* 1980, Darise *et al.* 1983, Hsing *et al.* 1983). In addition, the results obtained in the above mentioned studies markedly differ from each other. Finally, there is only one report describing the changes in both the composition and content of the SGs in stevia shoots during different phases of plant development (Kang and Lee 1981). The aim of the present work was to characterise the distribution of the SGs in intact stevia plants during ontogeny.

Intact stevia (*Stevia rebaudiana* Bertoni, *Compositae*) plants purchased from the Mazlumov All-Russian Research Institute of Sugar Beet and Sugar (Ramone, Voronezh Region, Russia) were used. The experiments were carried out mainly on two stevia clones (0 and 28) possessing higher productivity in terms of their biomass and the SG content as compared to those of other clones of the same plant, but in some experiments five other clones of stevia were used. The plants were grown in glasshouse in soil mixture containing equal proportions of turf earth, sand and peat. Temperature varied from 26 to 35 °C. The experiments were performed during three vegetative periods (1998 - 2001). For the analysis of the SGs at each stage, ten plants were selected and used in the experiments. To test the distribution of the SGs in stevia leaves and stems the appropriate measurements were carried out on: *1*) young actively growing shoot section (shoot apex); *2*) mature shoot section (middle part

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Abbreviations: HPLC - high performance liquid chromatography; SGs - steviol glycosides.

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of shoot); *3)* senescent shoot section (lower part of shoot). The content of three major SGs (stevioside and rebaudiosides A and C) in plant samples was determined by HPLC after prior extraction and purification of such compounds.

Lyophilized and powered plant biomass (100 - 500 mg) was placed into centrifuge tubes, treated twice with 100 % methanol for 3 and 1 h on magnetic stirrer and centrifuged for 5 min at 240 *g*. The pooled supernatants obtained from each of the samples were dried under vacuum at 45 - 50 °C on rotor evaporator. Samples were purified by polypropylene *Sep-Pak* cartridges filled with sorbent *Separon*^{TM}*SGX C-18* (60 μm, *Tessek*, Prague, Czech Republic). The dry remainder of the sample extracts was dissolved in distilled water and loaded into the cartridge pre-activated with 2 cm^3 of acetonitrile and 2 cm^3 of distilled water. The cartridge was washed with 10 cm^3 of distilled water, 5 cm^3 of 20 % (v/v) acetonitrile - water mixture and finally 7 cm^3 of 80 % (v/v) acetonitrile - water mixture. The latter fraction obtained was evaporated under vacuum at 50 °C on rotor evaporator. The dry remainder was dissolved in 85 % (v/v) acetonitrile - water mixture and the solution obtained was passed through the microfilter with a pore diameter of 0.2 μm.

This assay was performed on a chromatographic apparatus (*LKB*, Bromma, Sweden) with 10-mm^3 calibrated loop, detection at 210 nm. The samples were chromatographed on steel *Ultra Pak* column (*TSK-OH-120*, 4.6 × 250 mm) with particle size of 5 μm using 85 % (v/v) acetonitrile - water mixture at flow rate of 0.5 cm^3 min^{-1}. Quantification of the fractions tested was carried out on the base of the calibrated data obtained with the use of standard SG samples purchased from Arbuzov Institute of Organic and Physical Chemistry of RAS (Kazan, Russia).

The obtained data are presented as the means of the measurements from 2 - 3 separate experiments.

The highest total content of the SGs was detected in leaves, whereas their content in flowers and stems was 7 - 8 and 12 - 13 fold lower, respectively (Fig. 1). In seeds the amount of the SGs was 2 - 2.5 times lower than that in flowers. The roots were found to have the lowest content of the SGs that is less than 0.1 % of root dry mass and more than 40 times lower than that in leaves. The tested organs of stevia contained all three major SGs. The proportions of the above SGs in these organs were found to be different. Thus, stevioside prevailed in flowers and seeds while the content of rebaudioside C was the lowest. In roots the content of all SGs was approximately equal, in stems such proportion was found only between stevioside and rebaudioside A, whereas the rebaudioside C content was always significantly lower. In leaves the SG proportions might be greatly changed depending on plant age and a phase of plant development (Fig. 2*A*,*B*).

The fact that the SG content in stevia leaves appeared to be substantially higher than that in flowers of this plant is generally in accordance with the data obtained by Zaidan *et al.* (1980) in respect to stevioside. According to this report the ratio in question appeared to be about 2.5, whereas our results showed that this value achieves about 7 - 8. On the other hand, as follows from the same report, only trace amounts of stevioside were found in stevia stems, whereas in roots the presence of this glycoside was not found at all. An apparent disagreement of these results with our data can be explained by the fact that as described above we used a new procedure for purification of the extracts reducing the SG loss to a minimum.

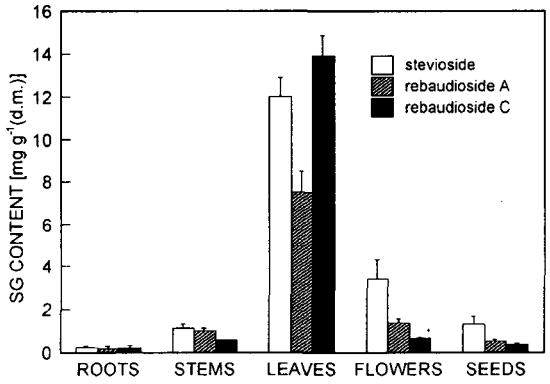

Fig. 1. The composition and content of the SGs in vegetative and reproductive organs of *Stevia rebaudiana* (clone 0). The SG content in the vegetative organs is presented at the end of the vegetative phase while that in the reproductive organs (flowers and seeds) at the appropriate phase of ontogeny.

In upper young, actively growing leaves the SG content was higher as compared to that in the same plant organs that finished their growth (Fig. 2*A*). The lowest content of the SGs was found in lower shoot section, namely in senescent leaves. In fact the same picture was revealed in the appropriate stem sections. It is interesting to note that younger leaves of *Scoparia dulcis* contained also higher amount of labdane and scopadulane type diterpenoids than older ones (Hayashi *et al.* 1991). According to our previous data lower mature leaves of stevia have smaller number of glands per leaf surface area than upper, younger leaves, *i.e.* positive correlation between the gland distribution density and the SGs content takes place. This argues in favour of possible accumulation of the SGs in the glands. The level of SG accumulation in upper young leaves as compared to lower senescent ones increased by 30 - 170 % depending on the clone, the portion of the rebaudioside A in the total SG content appeared to be increased as well.

During ontogeny the SG accumulation in above-ground vegetative stevia organs (mature leaves and stems) took place (Fig. 2*B*). Thus their content gradually increased up to the budding phase and even the onset of flowering. There is a report (Kang and Lee 1981) demonstrating that the maximal content of stevioside in

leaves is achieved during the formation of flower buds and then it gradually declines. All these data may indicate that the SGs are transported to generative organs. Similar results were obtained for ecdisteroids in *Rhaponticum carthamoides*, *Ajuga reptans* and *Serratula coronata* (Vereskovskiĭ *et al.* 1983, Revina *et al.* 1986, Tomas *et al.* 1993, Anufrieva *et al.* 1998). Here these compounds were shown to undergo a primary production in young leaves and subsequent accumulation in reproductive organs as well. The accumulation of the ecdisteroids occurred in those organs that are responsible for the preservation of the species under the given ecological conditions (Volodin 1999). For example, in *Rhaponticum carthamoides* and in *Serratula coronata* this accumulation took place in seeds and roots, while in *Ajuga reptans* it occurred in roots or in wintergreen leaves (Tomas *et al.* 1992, Volodin 1999). During ontogeny little variation of the SG content was found in roots as well (data not shown). In these organs, from the vegetative phase up to the flowering gradual decrease of the SG content was observed. During the fruit development stage the SG amount was found to revert to the initial level. However, in roots the total SG content never exceed 0.1 %. It is interesting to note that the same changes of ecdisteroid content were found in the roots of *Ajuga reptans* (Volodin 1999).

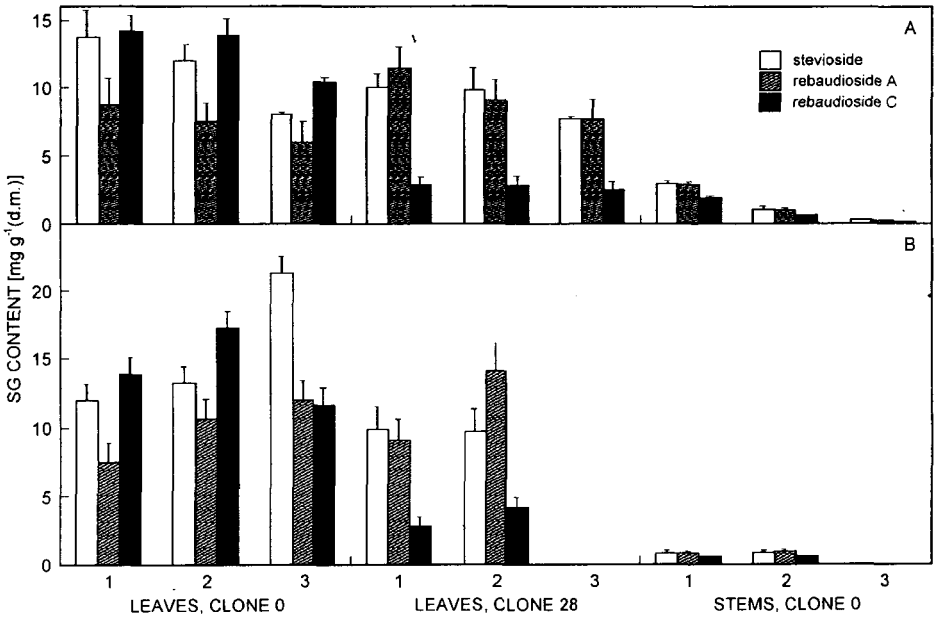

Fig. 2. The SG content in the aboveground vegetative organs of *Stevia rebaudiana*. *A*. 1 - upper young shoot sections; 2 - middle mature shoot sections; 3 - lower senescent shoot sections. The data presented were obtained at the end of the vegetative phase of plant development. *B*. 1 - vegetative phase; 2 - budding phase; 3 - onset of flowering. The SG content in mature shoot section is presented.

Our conclusion is that all the tested organs of stevia contain both the stevioside and rebaudiosides A and C, but the proportions of the SGs were different. In leaves the SG proportion might be greatly changed depending on plant age and a phase of plant development. Individual organs of stevia appear to significantly differ in the SG content and this value declines in the following order: leaves, flowers, stems, seeds, roots. The highest amount of the SGs is inherent in upper, actively growing shoot sections, whereas lower, senescent shoot sections have the lowest content of such compounds. During ontogenesis, leaves and stems of stevia gradually accumulate the SGs up to the budding phase and the onset of flowering. The highest content of the SGs in leaves suggests that namely leaves serve as a main organ for both synthesis and primary accumulation of such compounds.

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