Bulgarian Chemical Communications, Volume 49, Special Edition B (pp. 121 – 125) 2017

Content of steviol glycosides in stevia (*Stevia Rebaudiana* B.) genotypes cultivated in Bulgaria

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Received February 06, 2017; Revised February 24, 2017

Dedicated to Acad. Bogdan Kurtev on the occasion of his 100th birth anniversary

Current study presents data on the content of steviol-glycosides (stevioside and rebaudioside A) in twenty four genotypes of (*Stevia Rebaudiana* B.), cultivated in Bulgaria. The HPLC analysis showed that the content of stevioside in the separate groups reached 6.80 g/100 g dry mass and that of rebaudioside A – 8.27 g/100 g dry weight. These results allow us to make a conclusion that trough the path of individual selection, it is possible to develop stevia genotypes with high content of steviol-glycosides, under the climatic conditions of Bulgaria.

Key words: Stevia (*Stevia Rebaudiana* B.) leaves; HPLC determination; steviol glycosides; stevioside, rebaudioside A

INTRODUCTION

Stevia (*Stevia Rebaudiana* B.) is a perennial shrub of the *Asteracease* (*Compositae*) family, growing naturally in Paraguay and Brazil. Dried stevia leaves are a rich source of ent-kaurene-type diterpene glycosides, called steviol-glycosides and the most abundant of them is stevioside [1]. Being 300 times sweeter than sucrose, stevioside is considered as one of the strongest natural sweeteners [2]. The content of stevioside in stevia leaves is in the range 4–20% and depends on the cultivar and on growth conditions [3]. Other glycosides in the leaves are rebaudioside A (about 3%); rebaudioside C, D, E, F and dulcoside A (totally about 2%). Minor quantities of steviolbioside, rubusoside, and rebaudioside were found as well [4–7]. There are evidences that other steviol-glycosides such as steviolbioside and rebaudioside B could be formed by partial hydrolysis during the extraction process of steviol-glycosides [8, 9]. Except steviol-glycoside, stevia leaves contain variety of biologically active substances such as flavonoids, alkaloids, chlorophylls, xanthophylls, hydroxycynnamic acids, oligosaccharides, free sugars, amino acids, lipids and trace elements [10] and it is suggested that stevia extracts exert beneficial effects on human health, including anti-hypertensive anti-hyperglycemic and anti-human rotavirus activities [11–13].

For many years, food industry has traditionally used sugar as the main sweetening agent but in the last century many synthetic and natural sweeteners were released on the market as well. Nowadays, in response mainly to the consumer preferences, there is increasing demand for natural sweeteners. Among the mandatory requirements for the sweeteners such as lack of toxicity, consumers prefer sweeteners that are low in calories and have sugar-like taste profile. On the other hand, food industry requires sweeteners that are heat and pH stable. Steviol-glycosides being noncaloric and high potency sweetener fulfill these requirements and meet customer's preferences, and therefore subject of enormous interest in the recent years. The commercial exploration of Stevia has become stronger since the 70's, when Japanese researchers developed a series of processes for the extraction and refining of the leaf sweeteners. It was claimed that stevioside had a 20% of market share of low-calorie sweeteners in Japan [14]. Nowadays, steviol glycosides (stevioside and rebaudioside A) are approved for general food use in Australia, Argentina, Brazil, China, European Union, India, Israel, Japan, New Zealand, Paraguay, Russia, South Korea and few other countries. In the USA, rebaudioside A and highly purified steviol glycosides received status "generally recognized as safe". Presently stevia is recognized as a plant with a significant economic value [15, 16] and is being cultivated in different countries worldwide. Not surprisingly many countries conduct their own research directed to the selection of high steviol-

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glycosides cultivars and development of extraction and separation techniques for these natural sweet compounds [17].

Stevia is a relatively plastic culture. The development of the plant is influenced by the region and geographical latitudes [18]. The high plasticity of the plant is significantly influenced by the unique conditions of the area where it is cultivated [19]. The climate of Paraguay is subtropical and characteristic with variable temperatures and high amount of rainfalls. Stevia is thermophilic culture and it was found that temperatures below +12 °C suppress plant development. The plant is highly sensitive to low temperatures and genotypes, developed in Bulgaria freeze at 1-2 °C [18]. Therefore, under the climatic conditions of Bulgaria stevia can be grown as annual crop. The purpose of the current study was to determine the content of steviol-glycosides in 24 genotypes of Stevia (*Stevia Rebaudiana* B.), cultivated in Bulgaria. Such a comparative study is the initial step in the development of Stevia cultivar with high content of steviol-glycosides.

EXPERIMENTAL

Plant Materials

Stevia plants from different genotypes were cultivated on the experimental fields of the Agricultural Institute, Shumen on carbonate chernozem soils, under irrigation. Altogether twenty four stevia genotypes were studied. Genetic material used is included in the breeding program of the Institute and plants were derived and adapted in tissue culture laboratory. Ten individual plants of each origin were evaluated. Plants were harvested in the autumn and fresh leaves were collected from the stem. Leaves were dried in shade at room temperature. Dry leaves were stored in paper bags prior to analysis. The dry matter and the content of steviol-glycosides were analyzed in each sample.

Extraction of Steviol-glycosides

Dry stevia leaves were ground with a coffee mill to a homogeneous powder mass. About 1g of the ground mass was weighed accurately and extracted with 100 ml water at water bath (95 °C) for 30 min. The mixture was cooled to room temperature and filtrated trough filter paper. The filtrate was separated and the residue was subjected to a second extraction under the same conditions. Both supernatants were combined and the volume was adjusted to 200 ml with distilled water.

Extracts purification trough solid-phase extraction

Stevia extracts were subjected to purification by solid-phase extraction. For this purpose, 3 ml of the extract was passed through a column Oasis C-18, activated with pure methanol. Impurities in the sample were eluted first with 5 ml water and then with 5 ml 40% methanol (v:v). Steviol-glycosides, were eluted from the column using 5 ml of 70% methanol (v:v). The resulting fraction was used to determine the steviol-glycosides by high performance liquid chromatography (HPLC).

High performance liquid chromatography (HPLC) of steviol-glycosides

To determine the content of stevioside and rebaudioside A in stevia extracts an Agilent 1220 chromatographic system equipped with a UV-Vis detector Agilent 1220 was used. The elution was isocratic with a mobile phase acetonitrile:water (80:20). The separation of steviol glycosides, was performed on chromatographic column Agilent Zorbax Carbohydrate (4.6 x 150 mm, 5 µm) at room temperature and flow rate of the mobile phase, 1 ml/min at a wavelength of λ = 210 nm. External standards (stevioside and rebaudioside A) were used to quantify the content steviol-glycosides. All analyses were performed two times and their content was expressed in g/100 g dry leaf mass.

RESULTS AND DISCUSSION

As already mentioned the natural habitat of *S. rebaudiana* is located in the subtropics, but it was shown that the plant is worthy for cultivation in European countries [20, 21]. The increment of steviol-glycosides is the main goal in different breeding programs of stevia (*Stevia Rebaudiana* B.). It is known that long day conditions promote the vegetative growth and even increase steviol-glycosides in stevia [22]. This is an advantage of the European latitudes, compared to the shorter days in Paraguay and Brazil. It was proposed that the cultivation of *Stevia rebaudiana* could be economical feasible in Czech Republic [23] and Germany [24]. To our knowledge, the current study is the first report on the content of steviol glycosides in Stevia genotypes, cultivated in Bulgaria.

In our study, we investigated 24 stevia genotypes from the genetic bank of the Agricultural Institute – Shumen for their content of steviol-glycosides. The highest yield of a single plant has been found in genotype 2304, where the excess of the mean value for the group reached 37.5%. Genotypes 2101 and 2110 are characteristic with relatively equal results and with an excess of 28.7% and 30.2% respectively (Table 1).

Table 1. Content of dry matter in stevia leaves from different genotypes.

Genotype	Dry matter content, g	Relative value to the mean, %
2001	18.2	55.5
2002	31.8	97.0
2003	18.1	55.2
2101	42.2	128.4
2102	28.5	86.9
2204	31.5	96.1
2205	33.7	102.8
2206	26.9	82.1
2207	34.4	104.9
2210	42.7	130.2
2211	33.2	101.3
2212	37.2	113.5
2213	36.5	111.3
2301	29.5	90.0
2302	40.1	122.3
2303	31.9	97.3
2304	45.1	137.5
2305	25.3	77.2
2306	27.4	83.6
2401	27.5	83.9
242	31.7	96.7
2403	40.5	123.5
2404	31.1	104.0
2405	38.7	118.1
Mean	32.8	100

The HPLC method used by us allowed rapid and precise determination of steviol glycosides presented in the samples with very good separation of stevioside from rebaudioside A (Fig. 1).

From the data for individual stevioside content in the investigated selection materials, it is evident that the average stevioside content in the investigated genotypes was 6.38 g/100 g dry matter (Fig. 2). Genotypes 2304 and 2401 are characteristic with excess above the mean annual content in the range 39.5%–44.2%. It was found that the content of rebaudioside A, varies significantly among the investigated samples with average content 5.65 g/100 g dry weight. For example, genotype 2205 contains 3.3 g/100 g rebaudioside A, whereas its content in genotype 2303 reaches 16.5 g/100 g dry weight. The total content of sweet substances in the dry mass is a composite indicator comprising the amount of the two steviol-glycosides. Our results show that the average annual content of steviol-glycosides reaches 12.03 g/100 g. This parameter varies in relatively narrow range, indicating that to a larger extent this is a conservative feature. The highest content of total steviol-glycosides was recorded in genotype 2305, where the excess over the mean for the group was 83.7%, followed by genotype 2302 with 21.9 g/100 g (18.1%). These results allow us to make a conclusion that trough the path of individual selection it is possible to develop stevia genotypes with relatively high content of steviol-glycosides.

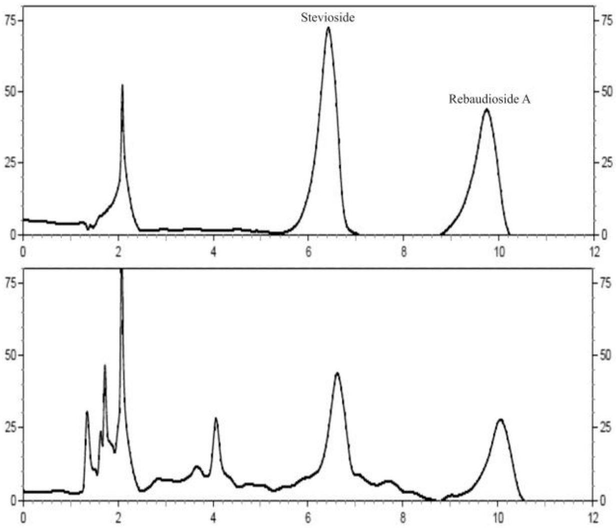

Fig. 1. HPLC chromatogram of 0.5 mg/ml pure steviol glycosides (upper panel) and extract from sample 2204 (lower panel).

Fig. 3 presents the average data for the groups of the studied genotypes. The highest stevioside content (average 6.71 g/100g) possess the material of origin 2400, whereas rebaudioside A content is the highest in the genotypes of group 2300 (8.27 g/100 g).

Rebaudioside A is the most desirable component due to its sweetening potency and superior taste profile [25]. Responding to this challenge, new cultivars with significantly higher concentration of rebaudioside A have been reported [26]. The native ratio stevioside:rebaudioside A in stevia leaves is usually about 2. As a result of the higher content, stevioside imparts a characteristic bitter after-taste

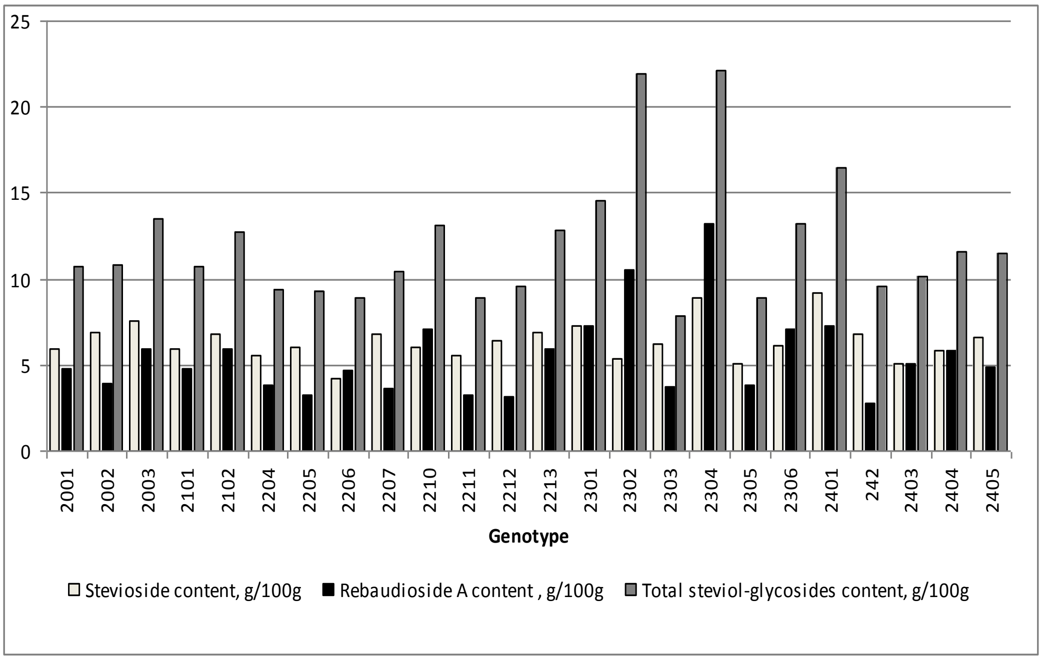

Fig. 2. Stevioside, rebaudioside A and total steviol-glycosides content in the leaves of different stevia genotypes.

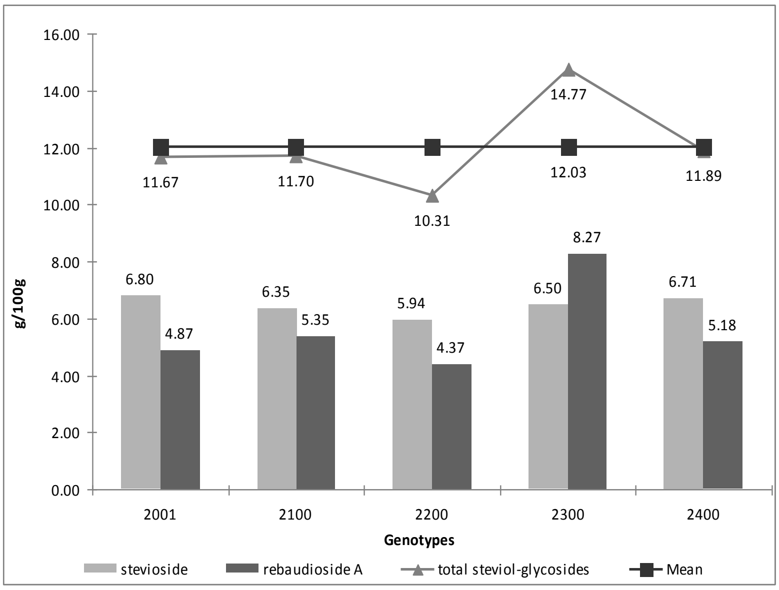

Fig. 3. Average content of stevioside, rebaudioside A and total steviol-glycosides content in the groups of the investigated genotypes.

to the crude extracts. Conversely, the most valuable extracts are those that have rebaudioside A as the major component, because of its organoleptic and physicochemical features (the best taste profile relative and the best solubility in water) [25], what permits a greater variety of formulations. Therefore, the phytochemical characterization of new genotypes and varieties of stevia with higher levels of total steviol-glycosides and particularly of rebaudioside A the main goal to research groups dealing with the improvement and utilization of this source of natural sweeteners. In our study the ratio between stevioside and rebaudioside A reached 1.13.

CONCLUSION

We investigated 24 stevia genotypes from the genetic bank of the Agricultural Institute – Shumen for their content of steviol-glycosides (stevioside and rebaudioside A). Genotypes with relatively high stevioside (2101 – 9.2 g/100 g) and rebaudioside A (2304 – 13.2 g/100 g) content were selected. The content of steviol-glycosides in the selection materials of genotype 2300 reaches 14.77 g/100 g dry weight. These results allow us to make a preliminary conclusion that under the climatic conditions of Bulgaria, it is possible to obtain stevia genotypes with relatively high content of steviol-glycosides. This opens the possibility to develop stevia cultivar in Bulgaria rich in steviol-glycosides and particularly in rebaudioside A.

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СЪДЪРЖАНИЕ НА СТЕВИОЛ ГЛИКОЗИДИ В ГЕНОТИПОВЕ СТЕВИЯ (*STEVIA REBAUDIANA* B.), КУЛТИВИРАНИ В БЪЛГАРИЯ

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Постъпила на 06 февруари 2017 г.; Коригирана на 24 февруари 2017 г.

(Резюме)

Настоящото изследване представя данни за съдържанието на стевиол гликозиди (стевиозид и ребаудиозид А) в двадесет и четири генотипа стевия (*Stevia rebaudiana* B.), отглеждани в България. Посредством високоефективна течна хроматография бе установено, че съдържанието на стевиозид в отделните групи достига 6.80 g/100 g суха маса, а това на ребаудиозид А – 8.27 g/100 g сухо тегло. Тези резултати позволяват да се направи заключение, че при климатичните условия в България, посредством пътя на индивидуален подбор е възможно селектиране на генотипове стевия с високо съдържание на стевиол гликозиди.