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Analysis of content of steviosides as biologically active compounds in stevia (*Stevia rebaudiana*) and products manufactured on the basis of this plant

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ABSTRACT

Stevia rebaudiana Bertoni is a desirable sugar substitute due to the presence of natural sweeteners (steviol glycosides). In 2011 steviol glycosides from this plant were accepted by the European Food Safety Authority (EFSA) as a harmless sweetener added to foods.The plant contains 9 different steviol derivatives commonly referred to as steviol glycosides. The largest share among them are: stevioside and rebaudioside A (the main components responsible for the sweet taste of the plant), while the others are rebaudioside B, C, D, E, F, dulcoside A and B and steviolbioside. The highest concentration of these substances is observed in the leaves. Stevia leaves may differ in the content of steviosides depending on the source. Therefore, the aim of the study was to compare the content of steviosides in stevia leaves (*Stevia rebaudiana*) from different countries and in products produced on the basis of this plant. On the basis of stevia leaf analysis, it was shown that the content of steviol glycosides in stevia leaves - rebaudioside A and stevioside, depends on the place and conditions in which it is grown. In addition, the amount of these substances in products based on this plant was analyzed. The study showed that they can be a substitute for dried leaves provided that the recommended daily intake is not exceeded.

Keywords**:** *Stevia rebaudiana* Bertoni, steviol glycosides, stevioside, rebaudioside A

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1. INTRODUCTION

Stevia comes from South America, but is now grown in many regions of the world. Among Stevia species, *Stevia rebaudiana* Bertoni has the highest sweetening potential. Due to its sweet taste, *Stevia rebaudiana* Bertoni has been the focus of interest for food manufacturers around the world from many years, usually as a natural sweetener added to food and beverages. The main purpose of its use, is to lower the energy value of the product by reducing the content of simple sugars. Observed excessive consumption of them is currently an increasing problem, because it may contribute to the development of health problems such as obesity, diabetes, atherosclerosis or hypertension [1].

There are 9 different steviol derivatives commonly referred to as steviol glycosides in *Stevia rebaudiana* Bertoni. The highest proportion among them is about 65% stevioside, about 25% rebaudioside A, while the others are rebaudioside B, C, D, E, F, dulcoside A and B and steviolbioside. The percent content of the above-mentioned steviol glycosides present in the total dry weight of stevia leaves is 5-10% for stevioside, rebaudioside A 2-5% and C 1%, dulcoside A 0.5%, rebaudioside D, E and F 0.2% and steviolbioside 0.1% [2].

The stevioside, originally called Eupatorin, is the main ingredient responsible for the sweet taste of stevia leaves. It is a white, crystalline and highly hygroscopic powder, and therefore must be stored in hermetically sealed packaging. The stevioside is completely natural, free of calories, and moreover 200 to 300 times sweeter compound than sucrose, commonly used to sweetening [3].

Rebaudioside is, in terms of quantity, the second glycoside found in stevia leaves. Like the stevioside, it has sweetening potential. It is characterized by a much better taste profile - after consumption, there is no tart flavor in the mouth, often referred to as the "licorice flavor" that appears in the case of stevioside [4].

The specific bitter taste of the stevioside is undetectable when the appropriate ratio of rebaudioside A to stevioside is maintained. Undesirable taste sensations may also be associated with residual contamination after extraction and purification.The most important feature of glycosides contained in stevia is the mouthfeel of a sweet aftertaste for quite a long time after consumption, which is much more intense and longer felt than after sucrose or aspartame [5].

In the human body glycosides are absorbed in the final part of the digestive system. Their decomposition to glucose and steviol occurs in the large intestine under the influence of bacteria, mainly *Bacteroides*. Metabolism products enter the bloodsystem through simple diffusion or active transport. Released from steviol glycosides, glucose is not absorbed into the body, but is consumed by bacteria living in the intestine. The absorbed steviol is transported to the liver, where it is converted to glucuronide. It is excreted in this form with urine or feces [1, 6].

Stevia as a sweetener may be used in many ways: concentrates, extracts as well as fresh and dried leaves (whole or powdered). Glycosides are also used to produce sweeteners in the form of juice from leaves, tablets, powder, liquid, but also concentrated extract of steviol glycosides in the form of a solution [7].

These glycosides in aqueous solutions shows good technological properties. They are resistant to sunlight, they can be heated to high temperatures (200 °C) in a wide pH range from 2 to 9. The stevioside is characterized by good stability at 120 °C with heating for one hour. Its complete degradation occurs only after exceeding 200 °C. Thus, it is possible to add this glycosides to food products requiring high temperature treatment, e.g. pasteurization, sterilization, cooking or baking without any effect on the product [8].

In November 2011, the European Food Safety Authority (EFSA) assessed steviol glycosides contained in the leaves of *Stevia rebaudiana* Bertoni as a safe food additive. It also specified the acceptable daily intake (ADI) of steviol glycosides of 4 mg per kilogram of body weight per day given as steviol equivalents. These substances are marked on the list of food additives with the symbol E960 [1, 9].

The protein content in stevia leaves ranges from 11.2 to 20.4 g/100 g of dry matter. Stevia leaves are a good source of carbohydrates and fiber. Carbohydrates according to research reach a value between 35.2 and 61.9 g/100 g dry matter. The content of fiber in stevia leaves is from 15.2 to 15.5 g/100 g dry matter. In stevia leaves there is from 1.9 to 4.34 g/100 g of fats in dry weight. According to research by Tadhani et al. [10] the largest share in their structure is palmitic acid (27.51%) and linoleic acid (21.5%). Due to the very low intake of this plant, nutrients such as carbohydrates, proteins and fats have a negligible effect on the body.

In Stevia leaves, there were also found ingredients such as calcium, phosphorus, sodium, potassium, iron, magnesium, zinc [4, 10-12]. In addition, according to research by Tadhani et al. [10] trace amounts of elements such as copper, cobalt, iron, manganese, zinc, selenium and molybdenum were also detected.

In the dried stevia leaves, 13 vitamins were found: 4 fat-soluble (A, D, E and K) and 9 soluble in water (B vitamins and vitamin C) [13].

It is believed that steviol glycosides, in addition to sweetening properties, also have health properties. The results presented in many studies confirm that compounds contained in the leaves of stevia can be used in the treatment of hypertension and diabetes. Research of Savita et al. [14] shows that stevia products affect blood sugar level, insulin levels, blood pressure, urinary sodium excretion, lipid profile and the weight. Research confirms that compounds contained in stevia reduce the level of sugar in the blood, which is important for people with diabetes [14, 15]. The results also show a weight loss of several kilos within 30 days [14]. Analysis of the influence of stevia on the change in body weight has shown that its addition to water administered to rats causes a significant reduction (over 40%) of body weight. Weight reduction obtained through the use of natural sweetener in the diet that is stevia, may be helpful in the treatment of overweight people and with type II diabetes [16].

According to research by Gregersen et al. [17], the stevioside reduces postprandial glucose level in the blood and probably contributes to increase in insulin secretion in patients with type II diabetes. The study of the effect of stevia consumption on the type II diabetes was made on a group of people diagnosed with diabetes. Patients received a 1 g of stevioside with a meal. The blood glucose level in patients with stevioside was 18% lower. Stevioside also contributed to the reduction of glucagon level after meals, thus positively influenced the glucose metabolism in people with type II diabetes. However, no effect of stevioside administration on the content of triglycerides and free fatty acids in the blood of patients was observed.

Research conducted on a group of 31 people by Anton et al. [18] shows that stevia, used as an additive to the diet, significantly lowers blood glucose level compared to the use of sucrose, as well as causes an increase insulin concentration after meal to a greater extent than when administering aspartame or sucrose.

In addition, positive effects of stevia on blood pressure have been found. The hypotensive effect of stevia is probably due to inhibition of the inflow of calcium ions into the cells, as a result of which the possibility of muscle contraction is blocked, the consequence of which is their relaxation. Relaxed vessels expand and facilitate blood flow, resulting in lowering blood pressure. The use of stevioside for at least 3 months results in a decrease in systolic and diastolic blood pressure. The therapeutic effect persists for a longer period of time after the completion of dietary supplementation with stevioside. Stevia doses, thanks to which blood pressure lowering is evident, reach values in the range of 750 to 1500 mg per day. In addition, the administration of steviosides has no negative effect on blood biochemical parameters and is usually well tolerated by patients [19]. After 30 days of administration steviol glycosides to patients, the average level of total cholesterol in the plasma as well as its HDL and LDL fractions also decreased.

Lowering cholesterol level in the blood may improve the health of people with atherosclerosis [14]. Research by Abu-Elnaga et al. [16] conducted on a group of 60 female rats confirms that stevia significantly reduces the level of total cholesterol and changes the level of its fractions. The LDL value in rat blood serum decreased with increasing dose of stevia administered with water. The largest decrease in LDL (26.5%) was recorded at a dose of 1000 mg/kg body weight/day. With the remaining doses, the decrease was lower: (500 mg/kg/day) by 24.36%, (250 mg/kg/day) by 19.90%, (25 mg/kg/day) by 15.01%. Unlike LDL, high-density lipoprotein HDL increased with increasing dose of stevia given to rats: (25 mg/kg/day) by 8.57%, (250 mg/kg/day) by 19.13%, (500 mg/kg/day) by 21.43%, (1000 mg/kg/day) by 24.73%. The dose of 25 mg/kg/day did not significantly change the HDL level in the serum compared to the control group and the group receiving sucrose.

There is an evidence that steviosides have anti-inflammatory effects both in vitro and in vivo. Initially, it was noted that topical dermatitis induced by 12-O-Tetradecanoylphorbol-13-acetate (TPA), was inhibited by steviol glycosides including stevioside. Due to the fact that TPA is also known as a cancer-inducing agent in mammalian cells, further research has been done on the antitumor properties of the stevioside. Studies in mice have shown that the stevioside inhibits the promotion of TPA-induced tumor in a skin tumor. Stevia steviosides are also found to be useful because of their ability to prevent the effects of inflammatory response in patients, while in healthy individuals they can increase the number of monocytes, and thus strengthen the innate immunity of the body [19].

Steviol glycosides lower the risk of tooth decay. Due to the positive effects of glycosides on the oral flora, these compounds are used in many toothpastes, mouthwashes and breath freshening products. Products containing stevia contribute to the formation of a smaller amount of acid compounds responsible for the degradation of the enamel than in the case of sucrose and other sugars, including simple sugars. According to research by Mohammadi - Sichani et al. [20] alcoholic and acetone extract from stevia leaves significantly lowers the growth of *Streptococcus mutans* bacteria on agar. These bacteria are a natural component of oral flora and contribute to the development of tooth decay. Stevia also has anti-inflammatory properties. There is a lot of evidence that steviosides have anti-inflammatory effects both *in vitro* and *in vivo*.

Many literature data shows that *Stevia rebaudiana* Bertoni also has antioxidant properties. Research shows that metabolites contained in plants and present in the human diet are a very good source of compounds with antioxidant properties. These compounds include: polyphenols and flavonoids, anthocyanins, vitamins (A, C and E), selenium, glutathione, thiocyanates, chlorophyllins and indoles. They counteract the formation of free radicals in the body. Polyphenols are secondary plant metabolites that arise from the mechanisms of biosynthesis shikimic acid. These compounds are antioxidants, as are ascorbic acid and α-d-tocopherol and carotenoids. These substances have a health-promoting effect protecting the human organism from oxidative stress [21]. Flavonoids are a large and best known group of phenolic compounds present in nature.According to research carried out by Tadhani et al. [22], the total content of polyphenols in stevia leaves is 25.18 mg/g, and in the stem 35.86 mg/g of dry weight. The content of flavonoids in the leaves was 21.73 mg/g, while in the stem, 31.99 mg/g of dry weight. Both the total content of polyphenols and the presence of flavonoids were significantly higher in the stem compared to the leaves.

The available literature data shows that *Stevia rebaudiana* Bertoni has also antibacterial and antifungal properties. In a study carried out by Abou-Arab et al. [11] they analyzed the antibacterial and antifungal properties of extracts derived from leaves and callus of stevia. The study was carried out using bacterial species: *Listeria monocytogenes*, *Staphylococcus aureus*, *Psudomonas aeruginosa*, *Salmonella typhimurium*, *Bacillus cereus* and *Escherichia coli*, and mold fungi: *Aspergillus ochraceus*, *Aspergillus parasiticus*, *Aspergillus flavus*, *Aspergillus niger* and *Fusarium*.

The stevia antibacterial properties were tested using six different solvents: acetone, chloroform, methanol, hexane, ethyl acetate and water. The methanol extract showed the highest antibacterial properties against *L. monocytogenes*, *S. aureus*, *P. aeruginosa* and *B. cereus*. Equally effective inhibition against the above bacteria was found in the case of acetone extract. Extracts based on ethyl acetate and water did not show any bactericidal properties. The higher antibacterial effect in the case of methanolic and acetone extracts may be due to the greater dissolution capacity in these organic solvents.

Among species of mold fungi the largest inhibition zone was recorded in the case of methanol extract against *Fusarium* and *A. niger*. Regarding the other mold fungi, no major changes in growth inhibition were observed, which may be related to the solubility of active substances in the solvent. The largest zone of growth inhibition was noted for *A. ochraceus* using chloroform extract, acetone extract and ethyl acetate from stevia leaves [11].

Stevia leaves may vary in the content of steviosides depending on the source. Therefore, the aim of the study was to compare the content of steviosides in stevia leaves (*Stevia rebaudiana*) from different countries and in products produced on the basis of this plant.

2. MATERIALS AND METHODS

2. 1. Plant material

Materials for research were purchased at the turn of February and March 2017 in stationary stores in the city of Lublin (Poland). Stevia leaves from the fourth group come from private crop located in Łosice in the province Mazowieckie (Poland). The studies used dried stevia leaves from various companies and countries:

1 - shredded leaf, company "Nanga", country of origin: Paraguay,

2 - shredded leaf, "Bionature" company, area of origin: EU,

3 - powdered leaf, "Bionature" company, area of origin: EU,

4 - whole leaf, private cultivation, country of origin: Poland,

5 - sweetener, company "Krüger", country of origin: Germany,

6 - syrup, company "Steviola", country of origin: Germany.

2. 2. Extraction of steviosides

To determine the content of steviosides, the stevia leaves were weighed into screw cap dishes (about 0.25 g each), then 6 mL methanol was added and after that Ultrasound-assisted extraction was used for 10 minutes. The extracts were then filtered through syringe filters (0.45 μm - Nylon). The sweetening tablets were prepared in the same way (weights about 0.15 g). The syrup was diluted 50 times with methanol. The samples thus prepared were subjected to chromatographic analysis. The content of stevioside and rebaudioside was determined in the tested samples.

2. 3. Chromatographic analysis

Chromatographic analysis was carried out at 30 °C using a Shimadzu UFLC chromatograph. The content of stevioside and reubaudioside A was determined by means of a diode detector at wavelength $\lambda = 210$ nm. Optimal chromatographic conditions were obtained using isocratic separation using a Synergi 4u Fussion-RP 80A chromatographic column size 250 mm × 4.6 mm at a mobile phase flow rate of 1 mL/min. The mobile phase was a mixture of phosphate buffer at 10 mmol/L and pH 3.00 and acetonitrile (68:32, v/v).

3. RESULT AND DISCUSSION

Extracts obtained from stevia leaves contain many substances in their composition which illustrate the obtained chromatograms (Figures 1, 2, 3). The peak from rebaudioside A was observed with a retention time of 11.55 min, whereas the peak was derived from the stevioside with a retention time of 12.62 min. Depending on the place of plant origin, the ratio of rebaudioside A to stevioside was different. Stevioside content is most often higher, however, in the samples from Poland, the dependence was reversed. This may be due to the different climatic conditions in which the plant grew.

The chromatographic analyzes carried out shows that the amount of rebaudioside A varies statistically significantly depending on the country of origin of the dried material. A similar relationship was also observed at the second compound - stevioside. The highest content of rebaudioside A was characterized by leaves from own cultivation in Poland, while the smallest comes from other European Union countries. In the case of the stevioside, its highest value was noted in the sample originating from Paraguay, while the smallest one is characterized by leaves from European Union countries.

The smallest amount of these compounds was observed in the third sample. Leaves from this sample came from plants grown in the European Union (Table 1). The different content of rebaudioside A and stevioside in individual groups is probably due to climatic conditions, the substrate on which plants grew, the length of exposure to sunlight, the age of the plant and the time of leaf harvesting.

Chromatographic analysis showed that there was 19.4g of rebaudioside A in 100g sweeteners based on stevia, with no stevioside present. The information placed on the label by the producer about its absence is true. In addition, syrup analysis confirmed the presence of both compounds at a concentration of: rebaudioside A - 36.630 mg/mL and stevioside - 17.6 mg/mL.

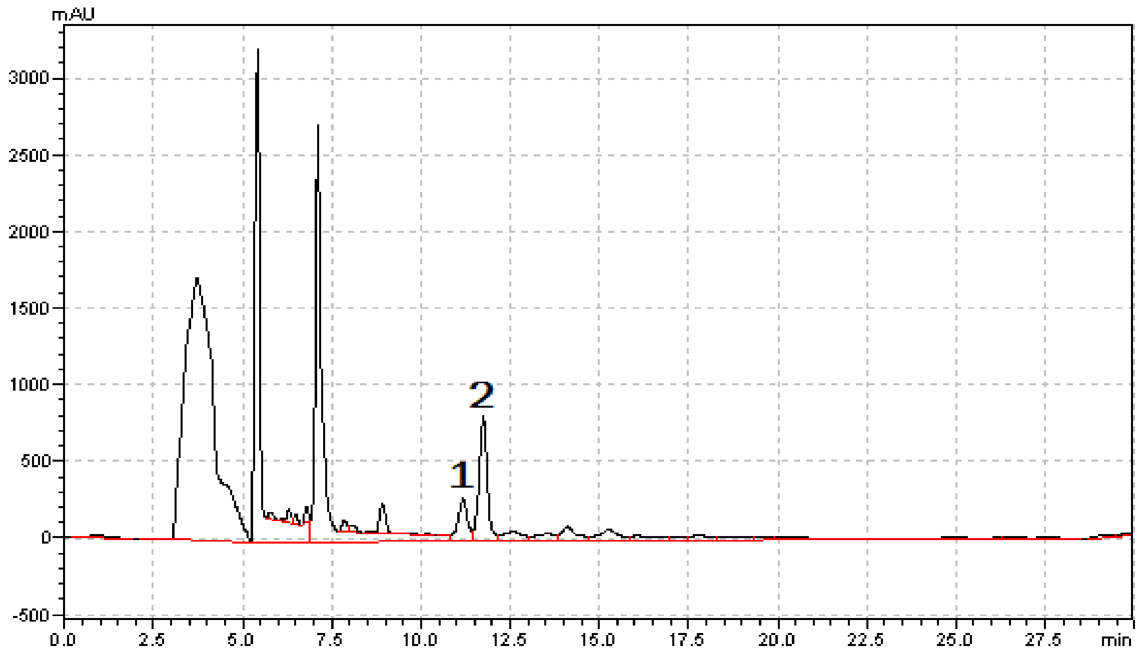

Figure 1. The chromatogram obtained during the analysis of shredded stevia leaves from European Union countries, 1 - rebaudioside A, 2 - stevioside.

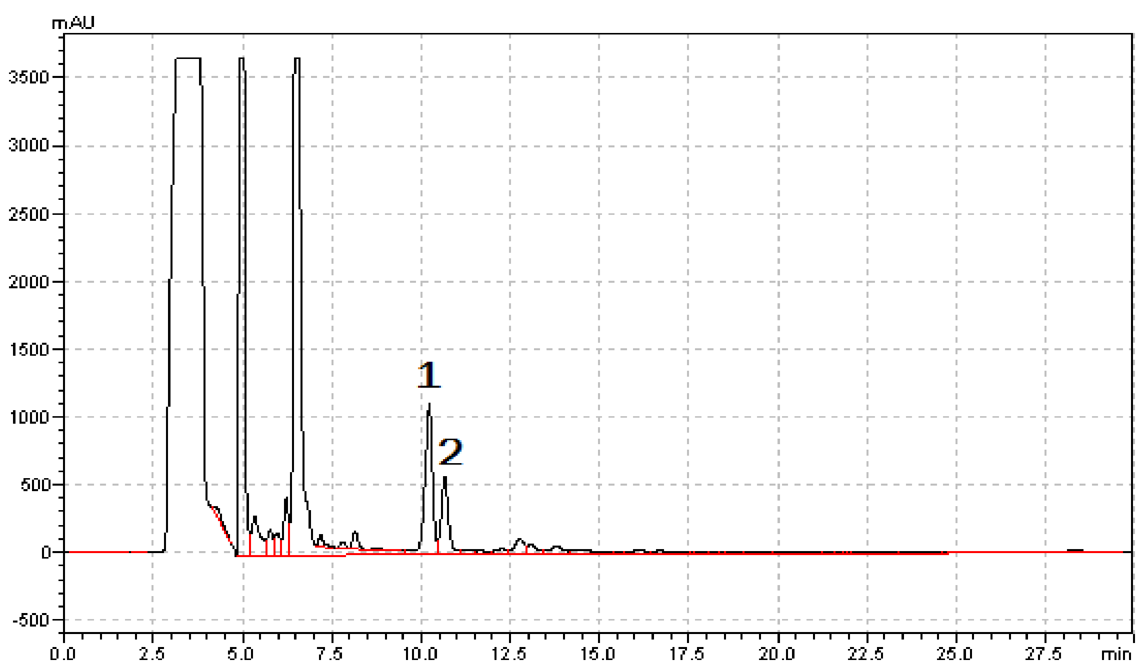

Figure 2. The chromatogram obtained during the analysis of whole stevia leaves from Poland, 1 - rebaudioside A, 2 - stevioside.

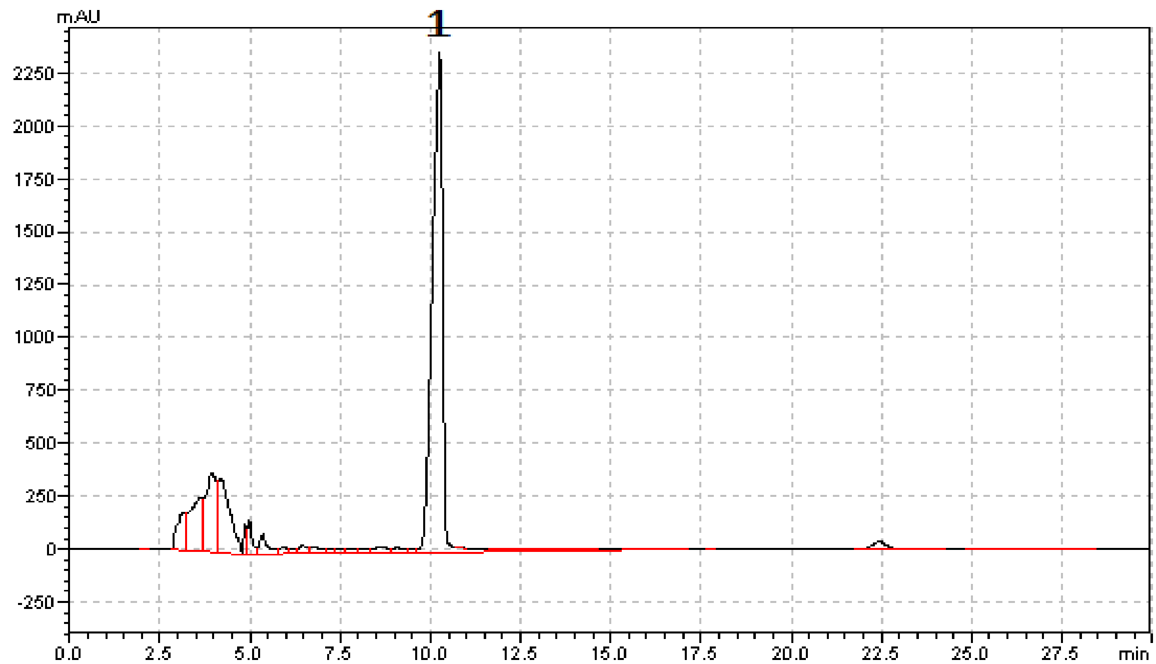

Figure 3. The chromatogram obtained during the analysis of the sweetener containing rebaudioside A, 1 - rebaudioside A

Table 1. Content of stevioside and rebaudioside A in stevia leaves.

Sample number	Rebaudioside A [g/100g]	Stevioside [g/100g]
1	3,309 [b]	4,084[a]
2	1,135[c]	1,516[b]
3	0,723[c]	0,951[b]
4	5,110[a]	1,155[b]

a, b, c - values marked with different letters were statistically different (P <0.05).

This study confirms the presence of the analyzed compounds in products containing steviol glycosides and the possibility of using them in small amounts while at the same time satisfying their performance. The characteristic bitter taste of the stevioside is undetectable when the appropriate ratio of rebaudioside A to stevioside is maintained.

The ratio desired by food producers depends on the conditions of plant cultivation and the time of leaf harvesting. The available literature data shows that the level of rebaudioside ranges from 1.8 to 5%, and the stevioside ranges from 4 to 13% in the dry weight. Table 2 presents the percentage of both compounds, according to different authors.

Table 2. Percent content of rebaudioside A and stevioside in dry plant weight based on available literature data.

Author	Rebaudioside A	Stevioside
Goyal et al. [3]	3,8%	9,1%
Ceunen et al. [2] Yadav et al. [5]	2 – 5%	5 – 10%
Lemus-Mondaca et al. [7]	2 – 4%	4 – 13%
Chatsudthipong i Muaprasat [19]	2 – 4%	5 – 10%
Gardana et al. [23]	1,8 %	5,8%
Atteh et al. [24]	2,3%	6,5%

The research carried out showed that in the commercially available dried stevia leaves from Paraguay and grown in Poland, the content of rebaudioside A is very similar to the value given by presented authors. In the case of the stevioside, the results indicate that in the stevia leaves grown in the European Union the amount of this compound is negligible in comparison with the results of the available literature data presented in Table 2. Higher content was recorded only in leaves from Paraguay. It may be a confirmation that the most favorable conditions for growing this plant in terms of content of steviol glycosides are present in South America, which is also place of its natural occurrence.

4. CONCLUSIONS

The content of steviol glycosides in stevia leaves - rebaudioside A and stevioside, depends on the place and conditions in which it is grown. The largest content of rebaudioside A was recorded in whole stevia leaves from own cultivation in Poland, and the smallest in ground leaves from other European Union countries. The highest content of stevioside was found in the leaves of stevia grown in Paraguay, while the smallest in leaves from the European Union.

References

[1] K. Marcinek, Z. Krejpcio, *ACTA Scientiarum Polonorum Technologia Alimentaria* 14(2) (2015) 145-152.

[2] S. Ceunen, J. M.C. Geuns, *Journal of Natural Products* 76 (2013) 1201-1228.

[3] S. K. Goyal, R. K. Samsher & Goyal, *International Journal of Food Sciences and Nutrition* 61(1) (2010) 1-10.

[4] M. Kobus – Moryson, A. Gramza – Michałowska, *ACTA Scientiarum Polonorum Technologia Alimentaria* 14(1) (2015) 5-13

[5] K. Yadav, S. Singh, D. Dhyani, P. S. Ahuja, *Canadian Journal of Plant Science* 91 (2011) 1-27.

[6] M. C. Carakostas, L. L. Curry, A. C. Boileau, D. J. Brusick, *Food and Chemical Toxicology* 46 (2008) 1-10.

[7] R. Lemus-Mondaca, A. Vega-Galvez, L. Zura-Bravo, K. Ah-Hen, *Food Chemistry* 132 (2012) 1121-1132.

[8] G. Kroyer, *Journal of Consumer Protection and Food Safety* 5 (2010) 225-229.

[9] Commission Regulation (EU) No 1131/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council with regard to steviol glycosides.

[10] M. Tadhani, R. Subhash, *Journal of Medical Sciences* 6 (2006) 321-326.

[11] A. Abou – Arab, M. F. Abu – Salem, *African Journal of Food Sciences* 4 (2010) 269-281.

[12] R. Kaushik, N. Pradeep, V. Vamshi, M. Geetha, A. Usha, *Journal of Food Science and Technology* 47 (2010) 27-33.

[13] Kim, M. Yang, O. Lee, S. Kang, *LWT – Food Science and Technology* 44 (2011) 1328-1332.

[14] S. Savita, K. Sheela, S. Suanda, A. Shankar, P. Ramakrishna, *Journal of Human Ecology* 15(2004) 261-264.

[15] N. Shivanna, M. Naika, F. Khanum, V. K. Kaul, *Journal of Diabetes and Its Complicaions* 27 (2013) 103-113.

[16] N. I. E. Abo-Elnaga, M. I. Masoud, M. I. Youself, H.H.A. Mohamed, *Annals of Agricultural Science* 61(1) (2016) 155-163.

[17] S. Gregersen, B. Jeppersen, J. J. Holst, K. Hermansen, *Metabolism* 53(1) (2004) 73-76.

[18] S. D.Anton, C. K. Martin, H. Han, S. Coulon, W. T. Cefalu, P. Geiselman, D. A.Williamson, *Apetite* 55 (2010) 37-43.

[19] V. Chatsudthipong, C. Muanprasat, *Pharmacology & Therapeutics* 121 (2009) 41-54.

[20] M. Mohammadi – Sichani, V. Karbasizadeh, F. Aghai, M. R. Mofid, *Journal of Medicinal Plants Research* 6(32) (2012) 4731-4734.

[21] C. A. Rice-Evans, N.J. Miller, G. Paganga, *Trends in Plant Science* 2 (4) (1997) 152-159.

[22] M. B. Tadhani, V. H. Patel, R. Subhash. *Journal of Food Composition and Analysis* 20 (2007) 323-329.

[23] C. Gardana, M. Scaglianti, P. Simonetti, *Journal of Chromatography A*, 1217 (2010) 1463-1470.

[24] Atteh, O. Onagbesan, K. Tona, J. Buyse, E. Decuypere, J. Geuns, *Archivos de Zootecnia* 60 (2011) 133-136.