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# Analysis of stevioside and rebaudioside A in sweeteners

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*ANALIZA STEWIOZYDU ORAZ REBAUDIOZYDU A W SŁODZIKACH*

*SUMMARY*

*Produkty z rośliny stewia mają coraz większe znaczenie, ponieważ wykazują korzystne właściwości żywieniowe i lecznicze. Głównymi związkami aktywnymi biologicznie, zawartymi w tego typu produktach, są dwa glikozydy stewiolowe: stewiozyd i rebaudiozyd A. Są one około 300 razy słodsze od sacharozy. Produkty obecne na rynku mogą istotnie różnić się jakością. W celu weryfikacji jakości słodzików ze stewii, opracowano nową procedurę analityczną. Wykorzystano metodę wysokosprawnej chromatografii cienkowarstwowej z detekcją densytometryczną, która zapewniła odpowiednią zdolność rozdzielczą, możliwość identyfikacji oraz oznaczania ilościowego. Proponowana procedura umożliwia dokonywanie oznaczeń w sposób prosty i bezpośredni, dając wiarygodne rezultaty. Celem pracy była ocena jakości kilku słodzików z uwzględnieniem zawartości stewiozydu i rebaudiozydu A, czasu rozpadu, masy i czystości tabletek. Rezultaty wskazują na znaczące różnice we właściwościach fizykochemicznych słodzików, co wpływa na ich właściwości sensoryczne i biologiczne.*

*KEY WORDS: STEWIOZYD – REBAUDIOZYD A – SŁODZIK – ANALIZA – JAKOŚĆ*

## Introduction

Stevioside and rebaudioside are *ent*-kaurene glycosides becoming form leaves of *Stevia rebaudiana* (Bertoni), the plant native to Paraguay and Brazil. Stevia has been used by Indians for centuries with tea or yerba mate to make the beverage sweet. The glycosides exert strong sweetness and are used in many low calorie sweeteners of natural origin. Nowadays, the high-scale usage of these compounds was initiated in Japan over 40 years ago, then it was cultivated in many countries all over the world (1-3). The leaves contain 4-20% diterpene glycosides (3, 4).

The Food and Drug Administration classified rebaudioside A as generally recognized as safe (5). The Joint FAO/WHO Expert Committee on Food Additives defined the admissible daily intake of stevioside as 0-10 mg/kg of body weight (6). In 2011 glycosides were accepted for usage in whole European Union as food additive E960 (7). The above mentioned substances have also many other beneficial values, they act as antihyperglycemic, hypotensive, antihyperlipidemic, antiinflammatory, antioxidant, antiviral, DNA-protective and chemopreventive agents (1, 4, 8-10). The further studies are required to justify that effects other than sweetness are clinically significant.

The glycosides from stevia are heat stable up to 200°C, so they can be used in variety of pastries. They are proper for cooking and baking. They also tolerate low pH values (were stable for 2 hours at 60°C in pH range 1-10) and do not undergo fermentation (3, 11). It is believed that they do not pose any allergic risk (3, 9). Chemical structure of stevioside and rebaudioside A are presented on figure 1.

Many chromatographic procedures, due to separation potential, were proposed for determination of steviol glycosides, including especially high-performance liquid chromatography HPLC (12-16) and thin-layer chromatography TLC (12, 15, 17-22).

TLC is a very useful and efficient tool in plant material analyses. It do not require high purification of analyzed samples, what is a necessity in column separation techniques. All hitherto published TLC procedures are laborious and require use of dying reagents. Because of lack of any simple, direct and effective high performance TLC procedure with densitometric detection, authors find it reasonable and valuable to develop a new procedure fulfilling these criteria.

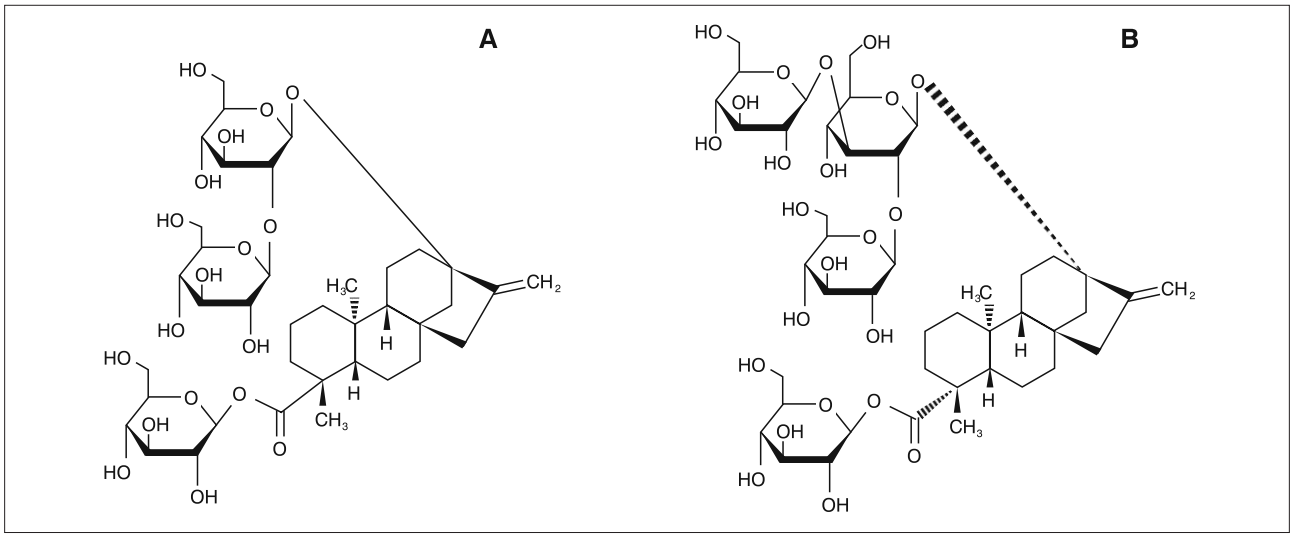


**Fig. 1.** Chemical structure of stevioside (A) and rebaudioside A (B).

## Materials and methods

### *Reagents*

All chemicals were of analytical grade and purchased from Chempur (Piekary Śląskie, Poland). Reference standards: stevioside hydrate (purity ≥ 98% by HPLC) and rebaudioside A (purity ≥ 96% by HPLC) were from Sigma-Aldrich (St. Louis, USA). Standard solutions, prepared with water-methanol mixture (1+4, v/v) with concentration of 2 mg/ml were used.

### *Material*

Five sweeteners coming from European Union market: Sweetiva Stevia, Süssina Stevia, Stevia Green Leaf, Stewia Planta Dulce and Stevija were investigated. The water-methanolic solutions were made. Milled tablet mass in amount 500 mg was extracted with mixture composed of 1 ml of bidistilled water and 4 ml of methanol for 15 min. The turbid solutions need to be filtered once (Stewia Planta Dulce trice) by filtration paper No 2.

Storage: the reference standards, standard and sample solutions were kept in a refrigerator at approx. 2-8°C protected from light. The sample solutions should be kept for no longer than 3 days. The sweeteners were stored in room temperature 15-25°C not exposed to the direct radiation.

### *Apparatus*

An 18 cm x 9 cm x 18 cm, Sigma Aldrich (St. Louis, USA) vertical chromatographic chamber, Linomat 5 band applying module and Scanner 3 densitometer were used. Additionally, a fluorescence detector at a wavelength of 254 nm was used in order to observe the quenching of fluorescence by the investigated substances what enable assessment of application correctness and preliminary separation efficiency. All three pieces of equipment were from Camag (Muttenz, Switzerland).

## Method parameters

### *Application*

The standard solutions and sample solutions were applied onto HPTLC silica gel 60 plates of 10 cm x 10 cm in size with F254 fluorescence factor (Merck, Darmstadt, Germany). The solutions were applied with a Linomat module with rate equal 200 nl/s. The bands were formed 10 mm from the bottom of the plate edge, while the front of chromatograms was fixed at 5 mm from the upper edge of the plate. There were six tracks on each plate. Band width was 7 mm and space between bands was 8 mm. The development distance equaled 85 mm.

The standard solutions were applied on chromatographic plate in volumes from 1.0 $\mu$l to 50.0 $\mu$l in order to establish method's analytical range which was determined as 7.5-22.5 $\mu$l for stevioside and 5.0-25.0 $\mu$l for rebaudioside A. In the case of sample solutions the application procedure was adjusted to method's range and required separate analysis of two analytes. The analyses of stevioside required bigger volums of sweeteners' solutions comparing with rebaudioside A. In case of Sweetiva Stevia, Süssina Stevia and Stewia Planta Dulce, there was applied 10 $\mu$l of solutions in order to determine stevioside and 5 $\mu$l for determination of rebaudioside A. Stevia Green Leaf and Stevija required 3 $\mu$l and 2 $\mu$l respectively.

***Separation***

The optimal mobile phase was established after many experiments. Finally, the chromatograms were developed with mobile phase consisted of ethyl acetate-methanol-water (7:2:1, v/v/v). The development of chromatograms took place in a closed saturated (5 min) chromatographic chamber. The development time equaled approximately 48 min.

***Densitometric detection***

The plates were scanned using the Scanner 3 densitometer in the UV range: 200-400 nm in the absorbance/reflectance mode. The slit dimension was defined as 6.0 mm x 0.1 mm. The scanning speed was 20 mm/s when recording densitograms and 100 nm/s when recording spectra. Scanning parameters were set using computer program winCats Planar Chromatography Manager. The source of UV radiation was a deuterium lamp. Scanning was performed at the wavelength $\lambda = 200$ nm, where the analytes maximum absorbance occurred.

All stages of analysis were performed in ambient temperature and pressure.

## Method validation

Analytical procedure, presented in this research has been validated in order to justify reliability of the method and results of the examination. The validation carried out according to ICH and Eurachem principles (23, 24) verified the precision, accuracy and linearity within the investigated concentrations range. The precision was measured by repeatability of the results at 100% of investigated level, (RSD for n = 9 results), while accuracy was expressed by the percentage recovery of the reference. The revovery was established for fruit methanolic extract (n = 6). Recovery was calculated using equation: recovery (%) = (C1-C2)/C3 X 100, where C1 is concentration determined in fortified sample, C2 is concentration determined in unfortified sample and C3 is concentration of fortification. The limit of detection (LOD) and the limit of quantification (LOQ) were derived from the ratio of signal level (s) to background noise (n) at s/n = 3 for LOD and 10 for LOQ.

Linearity was established for nine data points within the method's range with 5 $\mu$g intervals. The curvilinear dependence (quadratic equation) was observed, what is typical for densitometry (25). The specificity of the procedure was established based on the received values of retention factor $R_f$ and UV spectra. To examine robustness, in the course of procedure optimization, the importance of small variations of some parameters was investigated. This included altering the temperature (± 5°C), chamber saturation time (± 3 min), use of TLC (not HPTLC) plates, use of plates without F254 factor, volume of mobile phase (± 50%), manufacturers of methanol and ethyl acetate, drying conditions (ambient temperature or dryer, time), use of one-step distilled water instead of bidistilled. Influence of change of each parameter was investigated separately, keeping the other parameters untouched. The influence of these changes was really small and could be neglected. Use of 20 cm high plates instead of 10 cm and applying the spots, not bonds, do not alter the $R_f$ values. Samples storage time was found to be a significant factor, the samples should not be kept longer than 3 days in refrigerator due to precipitation of some components.

## Results

Under the established conditions, analyses were carried out. On the basis of recorded densitograms identification of stevioside and rebaudioside A as well as their quantitative determination were performed. The chromatograms developed with chosen mobile phase ensured favorable $R_f$ values for both steviol glycosides: for rebaudioside A approx. 0.35 and for stevioside approx. 0.45. The separation efficiency was good enough to perform the analysis and should be considered satisfactory, taking into consideration that difficult and laborious analysis of plant-derived material was done. The method is characterized by simple, one-step extraction process and enable direct determinations in UV light without need of derivatization and staining. Analitycal procedure presented in this thesis has been successfully validated.

Two representative densitograms are presented on figures 2 and 3. The validation parameters were presented in table 1, whereas results of determination were gathered in table 2.

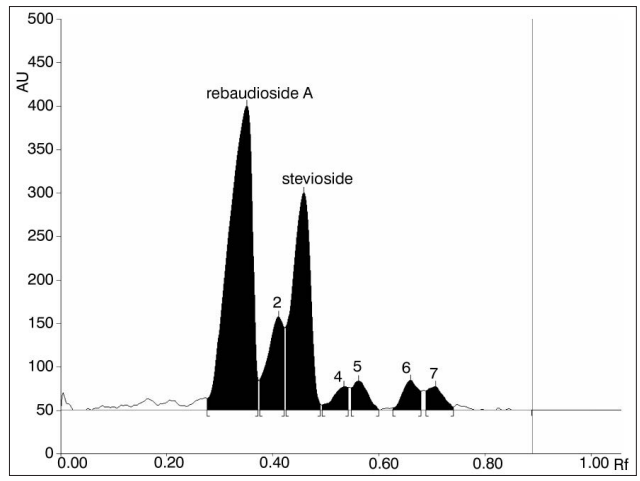


**Fig. 2.** Densitogram of extract from sweetener Stevia Green Leaf.

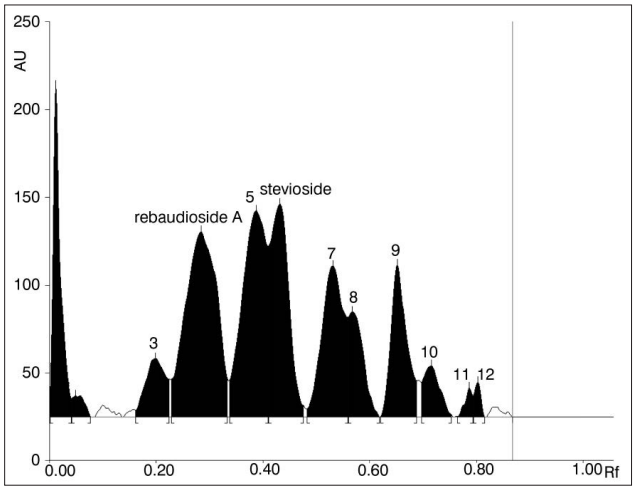


**Fig. 3.** Densitogram of extract from sweetener Stewia Planta Dulce.

## Discussion

It could be clearly seen on the figures that the dominant steviol glycosides present in the commercial sweeteners are rebaudioside A and stevioside. The less purified extract used the content of other glycosides higher. Table 1 presents content of analyzed substances in five investigated sweeteners: Sweetiva Stevia, Süssina Stevia, Stevia Green Leaf, Stewia Planta Dulce and Stevija. Four of products were white tablets, only Stewia Planta Dulce was yellowish. It is probably due to incomplete purification process of plant material used, what could be justified among others by presence of a peak at the start of chromatogram (yellowish stain on plate) (fig. 3).

The tablets of all sweeteners have similar weight, but have different disintegration time (conditions: bidistilled water, room temperature). In the case of three sweeteners there was possibility to analyze both compounds. For Stewia Planta Dulce determination was possible only for rebaudioside A, while stevioside could not be determined quantitatively due to significant interferences. In the case of Stevija the analysis demonstrate lack of stevioside peak. Rebaudioside A content is proved to be generally greater than stevioside. The biggest content was noticed in Stevia Green Leaf, where the sum od two analyzed compounds equaled 18.58 mg/tablet. Interestingly for this product a manufacturer declare adequately a minimal steviol glycoside content on the level of 18 mg.

**Table 1.** Analytical method validation parameters.
AU – absorption units, RSD – relative standard deviation, LOD – limit of detection, LOQ – limit of quantitation, Q – content ($\mu$g)

| Parameter | Stevioside | Rebaudioside A |
|---|---|---|
| Mean ± SD | 15545.17 AU ± 121.94 | 13564.27 AU ± 253.61 |
| RSD (%) | 0.78 | 1.87 |
| Recovery (%) | 97.4 | 96.8 |
| Range ($\mu$g/band) | 15.0-55.0 | 10.0-50.0 |
| Quadratic equation | $AU = -2.027Q^2 + 373.803Q + 3625.614$ | $AU = -2.396Q^2 + 497.465Q + 751.436$ |
| LOD ($\mu$g/band) | 4.0 | 2.0 |
| LOQ ($\mu$g/band) | 12.5 | 7.5 |

**Table 2.** Quantitative and qualitative assessment of stevia sweeteners.

| Sweetener | Stevioside [mg/tablet] | Rebaudioside A [mg/tablet] | Tablet disintegration time [min] | Average tablet weight [mg] |
|---|---|---|---|---|
| Sweetiva Stevia | 2.17 | 3.40 | 2.55 | 55.3 |
| Süssina Stevia | 2.90 | 3.79 | 3.50 | 58.3 |
| Stevia Green Leaf | 7.56 | 11.02 | 55.00 | 51.7 |
| Stewia Planta Dulce | (determination impossible – interferences) | 1.79 | 8.30 | 57.1 |
| Stevija | no stevioside | 8.77 | 3.40 | 57.5 |

On the other hand this preparation has exceptionally long disintegration time 55 min what negatively influence bioavailability.

## Conclusions

In conclusion, a new, simple, rapid, high throughput and cheap HPTLC procedure with densitometric detection was developed for separation, identification and quantification of two main steviol glycosides: stevioside and rebaudioside A in different stevia-based sweeteners. It was the first thorough discussion on stevia sweeteners quality. The method is suitable for routine food quality testing. This is especially important because of the fact that stevia glycosides are in increasingly common use. The researches revealed differences in purification, composition of excipients and the contents of the main active components: stevioside and rebaudioside A. Various properties of tablets was also demonstrate basing on different disintegration times. The quality assessment indicates that differences in physico-chemical properties of sweeteners are significant and may alter the sensoric and biological effects.

### Piśmiennictwo

**1.** Buszewski B, Noga S. Hydrophilic interaction liquid chromatography (HILIC)–a powerful separation technique. Anal Bioanal Chem 2012; 402:231-47. **2.** Cardello HMAB, da Silva MAPA, Damasio MH. Measurement of the relative sweetness of stevia extract, aspartame and cyclamate/saccharin blend as compared to sucrose at different concentrations. Plant Foods Human Nutr 1999; 54:119-30. **3.** Lemus-Mondaca R, Vega-Gálvez A, Zura-Bravo L et al. *Stevia rebaudiana* Bertoni, source of a high-potency natural sweetener: a comprehensive review on the biochemical, nutritional and functional aspects. Food Chem 2012; 132:1121-32. **4.** Pawar RS, Krynitsky AJ, Rader JI. Sweeteners from plants – with emphasis on *Stevia rebaudiana* (Bertoni) and *Siraitia grosvenorii* (Swingle). Anal Bioanal Chem 2013; 405:4397-407. **5.** FDA. GRAS notice for rebaudioside A (REB A) No. 252, 2008. **6.** JECFA. Joint FAO/WHO Expert Committee on Food Additives Monographs 5, Compendium of Food Additive Specifications from 69th JEFCA Meeting, 2008. **7.** EU. Commission Regulation No 1131/2011, 2011. **8.** Baechler BJ, Nita F, Jones L et al. A novel liquid multi-phytonutrient supplement demonstrates DNA-protective effects. Plant Foods Human Nutr 2009; 64:81-5. **9.** Goyal SK, Samsher, Goyal RK. Stevia (*Stevia rebaudiana*) a bio-sweetener: a review. Int J Food Sci Nutr 2010; 61:1-10. **10.** Madan S, Ahmad S, Singh GN et al. *Stevia rebaudiana* (Bert.) Bertoni – a review. Indian J Nat Prod Resour 2010; 1:267-86. **11.** Šic-Žlabur J, Voća S, Dobričević N et al. *Stevia rebaudiana* Bertoni – a review of nutritional and biochemical properties of natural sweetener. Agric Consp Sci 2013; 78:25-30. **12.** Dacome AS, da Silva CC, da Costa CEM et al. Sweet diterpenic glycosides balance of a new cultivar of *Stevia rebaudiana* (Bert.) Bertoni: Isolation and quantitative distribution by chromatographic, spectroscopic, and electrophoretic methods. Process Biochem 2005; 40:3587-94. **13.** Gardana C, Scaglianti M, Simonetti P. Evaluation of steviol and its glycosides in Stevia rebaudiana leaves and commercial sweetener by ultra-high-performance liquid chromatography-mass spectrometry. J Chromatogr A 2010; 1217:1463-70. **14.** Kolb N, Herrera JL, Ferreyra DJ et al. Analysis of sweet diterpene glycosides from *Stevia rebaudiana*: improved HPLC method. J Agric Food Chem 2001; 49:4538-41. **15.** Nikolova-Damyanova B, Bankova V, Popov S. Separation and quantitation of stevioside and rebaudioside A in plant extracts by normal-phase high performance liquid chromatography and thin-layer chromatography: a comparison. Phytochem Anal 1994; 5:81-5. **16.** Woelwer-Rieck U, Lankes C, Wawrzun A et al.: Improved HPLC method for the evaluation of the major steviol glycosides in leaves of *Stevia rebaudiana*. Europ Food Res Tech 2010; 231:581-8. **17.** Chester K, Tamboli ET, Singh M et al. Simultaneous quantification of stevioside and rebaudioside A in different stevia samples collected from the Indian subcontinent. J Pharm Bioall Sci 2012; 4:276-81. **18.** Fullas F, Kim J, Compadre CM et al. Separation of natural product sweetening agents using over pressured layer chromatography. J Chromatogr 1989; 464:213-9. **19.** Jaitak V, Gupta AP, Kaul VK et al. Validated high-performance thin-layer chromatography method for steviol glycosides in *Stevia rebaudiana*. J Pharm Biomed Anal 2008; 47:790-4. **20.** Kedik SA, Fedorov SV, Yanul' NA et al. Medicinal plants. Chromatographic determination of stevioside in raw plant material. Pharm Chem J 2003; 37:19-22. **21.** Londhe SV, Nanaware SM. HPTLC method for simultaneous analysis of stevioside and rebaudioside-A in *Stevia rebaudiana*. J AOAC Int 2013; 96:24-6. **22.** Shirwaikar A, Parmar V, Bhagat J et al. Identification and estimation of stevioside in the commercial samples of stevia leaf and powder by HPTLC and HPLC. Int J Pharm Life Sci 2011; 2:1050-8. **23.** The fitness for purpose of analytical methods: A laboratory guide to method validation and related topics. Eurachem 1998. **24.** Harmonised Tripartite Guideline. Validation of analytical procedures: Text and methodology, Q2(R1). ICH 2005. **25.** Komsta Ł. Chemometric and statistical evaluation of calibration curves in pharmaceutical analysis – a short review on trends and recommendations. J AOAC Int 2012; 95:669-72.

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