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Variability of chemical composition and antioxidant activity of the raw material of the Stevia Rebaudia varieties during processing.

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The dissertation has been carried out at the Faculty of Natural Science and Health, Department of Chemistry, Batumi State University. Shota Rustaveli,

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Dissertation Experts:

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The dissertation work can be found in the library of Batumi Shota Rustaveli State University, as well as on the university website - www.bsu.edu.ge.

INTRODUCTION

The relevance of the thesis.

Georgia is a small country, therefore, the effective use of the special possibilities of natural and climatic conditions for growing local or introduced plants is especially important for the country. Today, unfortunately, in many cases, the soil is not used properly. It is cultivated with plants that are not studied or not suitable for environmental conditions. Crop production (leaf, fruit, etc.) is cost-effective when studying its chemical composition using modern physicochemical methods. The research is particularly relevant when it comes to plants containing biologically active compounds. Therefore, there should be established a qualitative and quantitative content, as well as the chemical structure and biological activity of a plant. There should also be studied the optimal period of accumulation of these compounds and, accordingly, the harvest time of raw material. It is important to adapt the most optimal (chemical composition, yield and other) varieties in this region. It is necessary to develop processing technology and pay attention to monitoring of biologically active compounds during the processing and in the obtained product. Early studies have shown that the Stevia plant of South American origin is particularly effective for soil and climatic conditions of western Georgia.

Plant Stevia (Stevia rebaudiana) is a perennial herb 30-60 cm tall, originally from Brazil and Paraguay. There are many synonyms for *Stevia*. In the language of the Guarani - the famous tribe of American Indians, this plant is called *Ka-Ji, Ka-A-Yupl, Ka-Ji-Heh*, what can be translated as "honey grass", "sweet plant"; it has been used in traditional dishes for over 1500 years. The leaves of Stevia contain 300 times sweeter than sugar low-calorie sweeteners - deterpenoid glycosides (steviazide, rebaudioside, etc.). Stevia is a natural non-carbohydrate sweetener with unique therapeutic and recreational properties.

In addition to the sweet glycosides, Stevia leaves contain many other substances that are beneficial to the human body. Unfortunately, artificial sweeteners are most common in Georgia, and Stevia is rarely used because it is less well known. In addition, the product imported from abroad, is relatively more expensive compared to other competitors.

Agro-environmental conditions of Georgia are well suited for the adaptation of Stevia, so its first plants appeared in the 80s of the 20th century.

The objectives and goals of the research are the following: to identify and study the biologically active compounds of plants obtained from the seeds of a new breed of Stevia, as well as the chemical analysis of the leaves of plants at all stages of growth-development; to determine the optimal period of harvesting; to develop the optimal conditions for drying and processing the leaves with the maximum preservation of the content of biologically active compounds; to develop a technology for the production of bioactive natural low-calorie sweeteners; to improve the technology of food production, with the resulting sweeteners; to choose technological regimes; to breed new Stevia varieties on small trial plantations (according to known agro-methods).

Scientific novelty. For the first time in Georgia, the qualitative and quantitative content of bioactive compounds contained in the leaves of unknown varieties of introduced Stevia, was studied using HPLC-UV, RI, Conductometry UP UPLC-PDA, MS, preparative and analytical columns, various sorbents and solvents, as well as other modern physical and chemical methods. As a result of the study, there have been isolated and identified 27 compounds and their quantitative content was determined. Using various methods, including superfluid extraction, preparations of various sweetness were obtained, and the technology for the production of consumer tablets was developed as well.

The practical significance of the work. There has been developed a technology for the production of low-calorie, vegetable sweetener with various sweetness and biological activity; the chemical composition of the plant material and the product derived from it, has been established as well. The possibilities of obtaining and drying the superfluid extract, the technological parameters of drying, using a spray dryer, have been studied.

The preparations and sweet tablets, which are 100, 200 and 300 times sweeter than sucrose (the so-called white Stevia), have been obtained. Their chemical composition was studied using HPLC and UPLC methods and various detectors.

Object, Material and Methods of Study: The research object is the various forms of leaves of the Stevia variety (*Stevia Rebaudiana Bertoni*), introduced in western Georgia, as well as preparations and tablets obtained after processing. The homeland of Stevia (*Stevia rebaudiana Bertoni)* is South America (Argentina, Bolivia, Brazil, Paraguay). This variety of Stevia was first described by the botanist Bertoni in 1899. Stevia belongs to the Asteracea family.

Within the framework of grant DO / 124 / 6-470 / 13 of the educational doctoral program for 2013–2014, there were acquired the seeds of various plant varieties (from Poland, Paraguay, Canada and other manufacturers), characterized by a high content of diterpene glycosides and high yield,

1. Honey Stevia (Stevia Rebaudiana) Herbal Plant! 10seeds *Natural Sweetener (Singapore)
2. >600mg DARK STEVIA SEEDS + FREE DRY LEAVES SAMPLE! SWEET LEAF KAHEE (Polish)
3. 3000 STEVIA REBAUDIANA SEEDS - Sweet Leaf seeds HIGH QUALITY High germination
4. 1500 ORGANIC NON GMO STEVIA REBAUDIANA SEEDS - Sweet Leaf High germination. (Stevia Rebaudiana Bertoni, Extremely sweet herb from Paraguay)
5. 1000 STEVIA REBAUDIANA SEEDS - Sweet Leaf High germination
6. Stevia Rebaudiana Seeds * 1g (2000 Seeds) * Stevia * Sweet Leaf * Sugar Herb * Flower * Garden
7. "Paraguay, motherland of Stevia"

The seeds were grown indoors (the greenhouse was equipped in accordance with the grant requirements), and the experimental plot was planted with standard seedlings. The seedlings were obtained from germinated seeds (introduced varieties) and by traditional grafting (for continuous and profitable production of seedlings in the future).

Pic. 1. Standard Stevia Crop

Harvesting, storage and further study of raw material Stevia occurred in different periods of the growing season, namely 2 months, 6 months of vegetation and flowering period (ripening). Various technological modes of drying of raw materials, including natural and artificial drying processes (natural, convection, combined, etc.) have been studied.

The possibilities of drying leaves under artificial conditions were identified and selected, and the temperature and duration of drying process were optimized, what made it possible to excludee degradation changes in sweet diterpene glycosides when drying raw Stevia leaves.

The following physic-chemical methods have been used for the research:

1. the preparations of biologically active compounds have been obtained by fluid extraction of spercritical pressure;
2. the individual compounds have been obtained by preparative chromatography;
3. The sweet diterpene glycosides have been identified with high efficiency and ultra high efficiency liquid chromatograph by HPLC-UV, RI, UPLC-PDA, MS method;
4. The phenolic compounds have been allocated and identified with HPLC-UV, RI, UPLC-PDA, MS method.
5. The quantitative content of diterpene glycosides and phenolic compounds with HPLC-UV, RI, UPLC-PDA, MS chromatography method.
6. The qualitative and quantitative content of cations has been determined with HPLC-conductivity methods.
7. The antioxidant activity has been determined (using stable radicals 2,2-diphenyl-1-picryl hydrazine) using DPPH method.
8. The quantitative content of flavonoids was determined by a spectral method ($AlCl_3$ - reactivate, based on routine calculation).
9. The number of common phenols was defined by Folin-Ciocalteu method (based on gallic acid calculation);
10. Water and dry substance were determined by refractometer-method.

11. The volatilization complex was defined by the gas chromatographic method (GC Thermo).

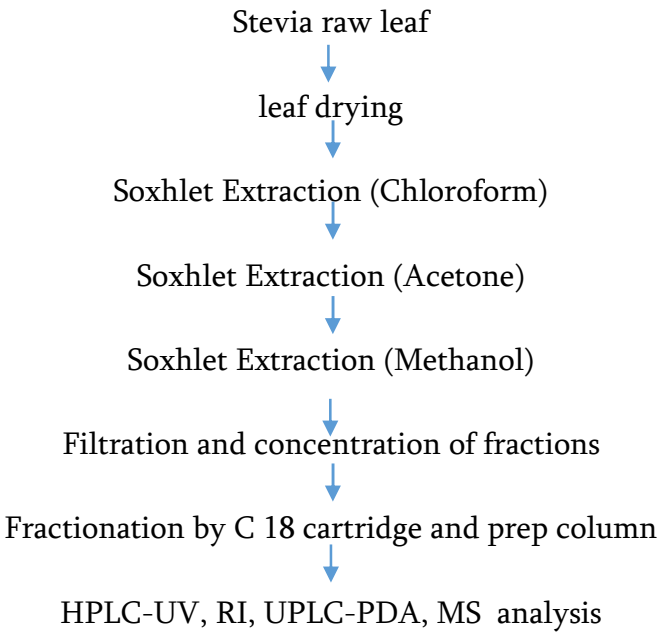
The approbation of research work. The results of the research are presented in 3 scientific articles and 5 international scientific conferences.

The volume and structure of the thesis. The dissertation consists of 112 printed pages, in accordance with the instructions of the dissertation submitted for the academic degree of Doctoral, and includes a title page and pages with signatures, resume in Georgian and English, content, a list of 15 tables, a list of 16 graphs, a list of references - 85 titles. The main text includes: introduction, literature review, analysis of the results, experimental part, conclusion, list of literature references and appendix.

Literature review - In the first chapter of the work deals with the Stevia plant, its bioactive compounds, sweet diterpene glycosides, the distribution of phenolic compounds in plants, their physiological activity and biological characteristics of forms of the Stevia plant, introduced to western Georgia. The dissertation is accompanied by a list of used literature references.

CHAPTER 1. Extraction and identification of Stevia's sweet diterpene glycosides

There has been used the following scheme for the extraction and identification of sweet diterpene glycosides of Stevia:



1.1.Steviol glycosides UPLC-PDA, MS analysis

The compounds have been extracted using high-performance liquid chromatography method and ultraviolet refractometry index detection, while their identification has been performed by ultra-efficient liquid chromatography UPLC and MS and PDA detectors. Substances were identified using standard compounds and the free data base https://metlin.scripps.edu of substance masses, as well as comparing data from peer-reviewed literary publications.

High pressure liquid chromatography (HPLC)- Waters (UV/Visible Detector 2489, Binary HPLC Pump 1525) chromatography column Symmetry C18, 3,5 μm 4,6 x 75 ∂∂, detecting 210 nm, solvent systems: Methanol (a), water (b) (4; 1), (Merck; Sigma-Aldrich) in linear gradient. Chromatography column amide (250 mm 4,5 mm), column temperature 40°C eluent 80% acetonitrile, RI detection.

Waters Acuity UPLC-PDA, MS, column BEN HSS (100x2.1 mm 1.7 μm). mobile phase 0.1 % Formic acid in DW (A), 0.1 % Formic acid in Acetonitrile (B), gradient solvent B gradient elution from 5% B for 1.5 min to reach 15% B at 4 min, 25% B at 25 to 16 min 65% B and 100% at 18,5-19.0 %, 0% B 19.0 to 20 min . Flow 0.3 ml min^{-1}, column temp 40 ^{0}C, MS-scan 40-1200 da, Probe 600 ^{0}C, Positive 0,8 kV, Capillary 1,5 kV, CV -40, PDA scan 210-500 nm.

The calibration curve of standard diperpene glycosides is constructed with 1.0, 2.0, 3.0 mg / ml concentration of 80% ACN / aqueous solvent of stevioside and rebaudioside (Sigma-Aldrich).

In order to construct the caliber curve of the injected sample of 3 μl, there have been used peak areas, formed for an individual compound, of the UPLC-MS system.

For sustained phase extraction (SPE) of Stevia's glycosides, 1.0 g of crushed leaves, pre-treated with chloroform in Soxhlet's device according to the scheme, were extracted by heating in an ultrasonic bath for 15 minutes, the extractant 50 ml ACN / water (70 : 30 volume). The obtained extract was filtered through 0.45 μm filter. SPE cartridges were filled with C18 sorbents

CHAPTER 2. The study of composition by the UPLC mass detector method

The research, identification and quantitative analysis have been carried out using UPLC-PDA-MS method. The method allows to investigate several compounds simultaneously; at the same time the reliability of their identification is quite high. There have been established chromatographic, spectral and mass spectral characteristics of the compound.

After concentrating the extracts, obtained by different solvent by SPE method, there was carried out chromatography using an amine preparation column (NH2, 5 μm, 250 × 10 mm).

A preparatory column was also used for chromatography (C18, 5 μm, 250 × 10 mm). There have been obtained 31 fractions. SPE cartridge was prepared (condensed) with water (1 ml) and 3 ml ACN / water (90:10); 1 ml of Stevia extract was passed through a cartridge; then Stevia glycosides were eluted with 2 ml of ACN / water (90:10). The obtained sample volume of 3 μl was injected into the LC-MS-PDA system. For hydrolysis of flavonoid glycosides, 5 mg of the preparation were dissolved in 2 ml of 2 M HCl and heated at 90 ° C for 40 minutes. In all cases, the analyzed extract was filtered through a 0.45 μm filter.

The fragmentation of compounds, as well as the change of their masses (at the expense of ions increase) and the maximum value of absorption in the UV area are very important for their identification.

The LC-MS-PDA study of diterpene glycosides allowed us to identify:
the following compounds:
the substance 1 - [M-H +] - m/z 319, [M-H -] - m/z 317, is observed on chromatogram in several places (at least 9 compounds, in accordance with all sweet diterpene glycosides), according to the compounds mass database METLIN (https://metlin.scripps.edu) (Appendix); the substance 1 corresponds to Aglikon Stevol and its isomer – isosteviol ($C_{20}H_{30}O_3$).

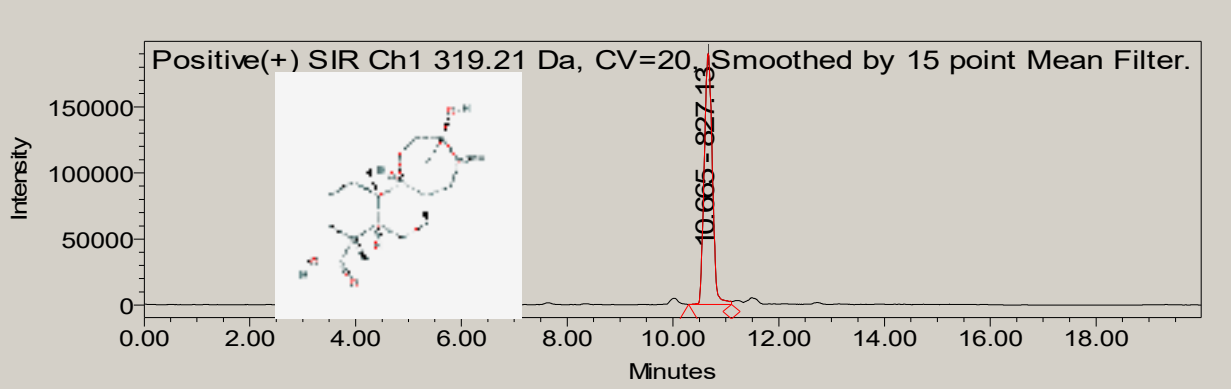


Fig. 1. UPLC-MS spectrum Steviol

the substance 2-[M-H -] - m/z 479, is observed on chromatogram - [M-H -] together with m/z 479. Retention time - 12.686 min, the maximum absorption - UV-211.9 nm. According to the compounds mass database METLIN, the substance 2 corresponds to steviol glycoside ($C_{26}H_{40}O_8$).

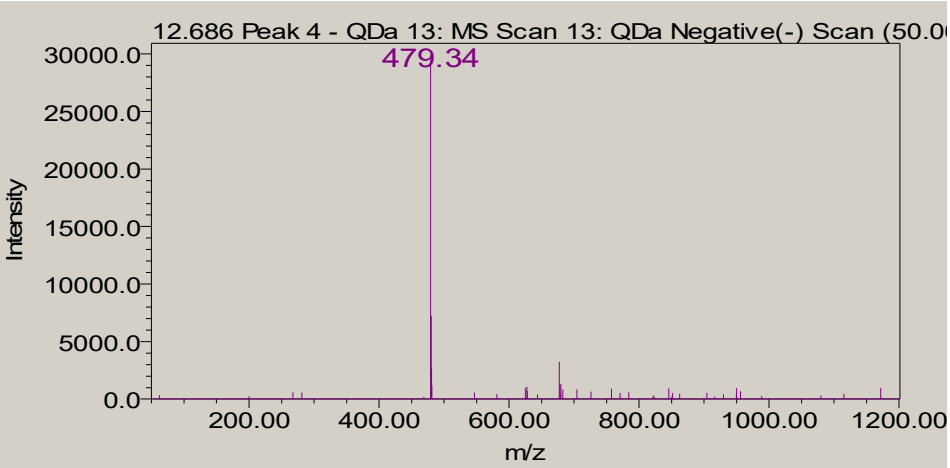


Fig. 2. UPLC-MS spectrum Steviol glycoside

The substance 3-[M-H -] - m/z 625, is observed on chromatogram -[M-H -] with m/z 787, 949. Retention time - 12.686 min, the maximum absorption - UV- 212.4 nm. According to the compounds mass database METLIN, the substance 3 corresponds to steviol diglycoside [M-16]($C_{32}H_{52}O_{14}$).

Fig. 3. UPLC-MS spectrum Steviol

The substance 4-[M-H -] - m/z 641, is observed on chromatogram -[M-H -] with m/z 803, 965. Retention time - 11.591 min, the maximum absorption - UV- 212.7 nm. According to the compounds mass database METLIN, the substance 4 corresponds to steviol bioside ($C_{32}H_{50}O_{10}$).

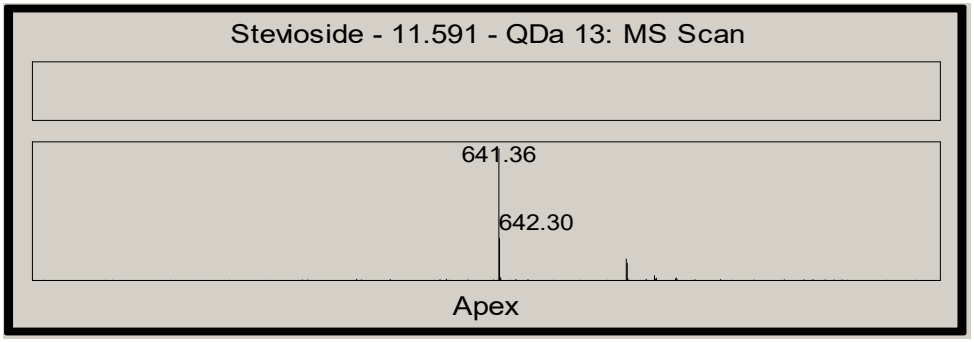


Fig. 4. UPLC-MS spectrum Steviol bioside

The substance 5-[M-H -] - m/z 787, is observed on chromatogram -[M-H -] with m/z 803, 965.

Retention time - 11.867 min, the maximum absorption - UV- 212.3 nm. According to the compounds mass database METLIN, the substance 5 corresponds to steviol triglycoside ($C_{38}H_{62}O_{19}$).

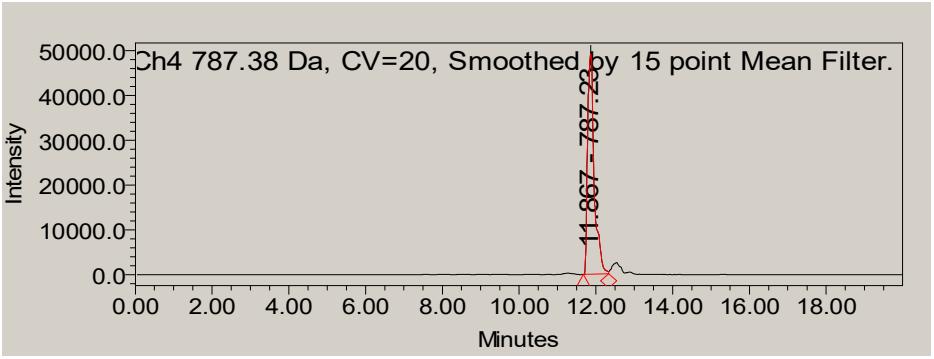


Fig. 5. UPLC-MS spectrum steviol triglycoside

The substance 6-[M-H -] - m/z 803, is observed on chromatogram -[M-H -] with m/z, 935,949, 965 or separately. The result of MS2 fragmentation is m/z 787([M-H + -2glc] -), while the result of the subsequent cleavage of glucose molecules MS3 is m / z 317 peaks (steviol). Retention time - 10.795 min, the maximum absorption - UV- 211.9 nm. According to the compounds mass database METLIN, the substance 6 corresponds to steviol triglycoside or stevioside (stevioside $C_{38}H_{60}O_{18}$) ([M-H +] - m/z 805.4). The result of stevioside fragmentation - ([M-H -] - m/z 641), while ([M-H +] - m/z 643). Also m/z 803, which is usually seen when chlorine ions (negative) and potassium ions (positive) are added; ([M-H^+ +K^+] - m/z 841), ([M-H -Cl^-] - m/z 839) and [M-H^+ -3glc] are formed respectively. The result of MS2 fragmentation from stevioside is the peak m / z 639 [M-H - -glc+ K^+], the glucose ([M-H2O]- m/z 162). This view has been documented as well

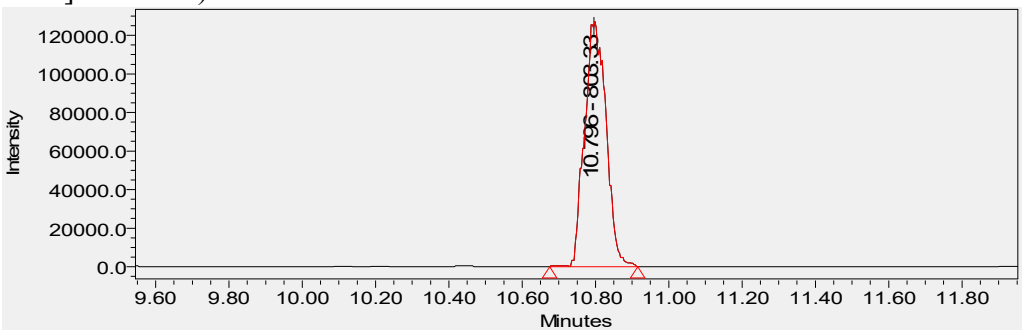


Fig. 6. UPLC-MS spectrum Stevioside

The substance 7-[M-H -] - m/z 965, is fixed on chromatogram -[M-H -] with m/z 803, 1001. Retention time - 10.798 min, the maximum absorption - UV- 212.9 nm. According to the compounds mass database METLIN, the substance 7 corresponds to steviol tetra-glycoside or rebaudioside A ($C_{44}H_{70}O_{23}$). Rebaudioside A ([M-H +] - m/z 965, the result of fragmentation MS2 is m/z 803, peak - [M-H + -glc], - glucose ([M-H2O]- m/z 162) from rebaudioside A. With the following fragmentation m/z 641([M-H + -2glc] -).

While rebaudioside A with the subsequent splitting of glucose molecules MS3 and MS4 is resulting into m/z 479 and m/z 317 peaks (steviol), [M-H + -3glc]- and [M-H + -4glc]- ions respectively. The addition of chlorine ions forms negative [M-H^+ +K^+] - m/z 1005, while the addition of potassium ions forms positive [M-H^+ -Cl^-] - m/z 1001.

During the chromatographic study of the standard rebaudioside A (Sigma-Aldrich), a few peaks are observed on the mass-spectrometer,-[M-H^+]- m/z 803,-[M-H^+]- m/z 965, -[M-H^-]- m/z 1001, as well as at a higher (more than 20 volt) charge -[M-H^+] - m/z 317 ѕб [M-H^+]$^+$ m/z 319.

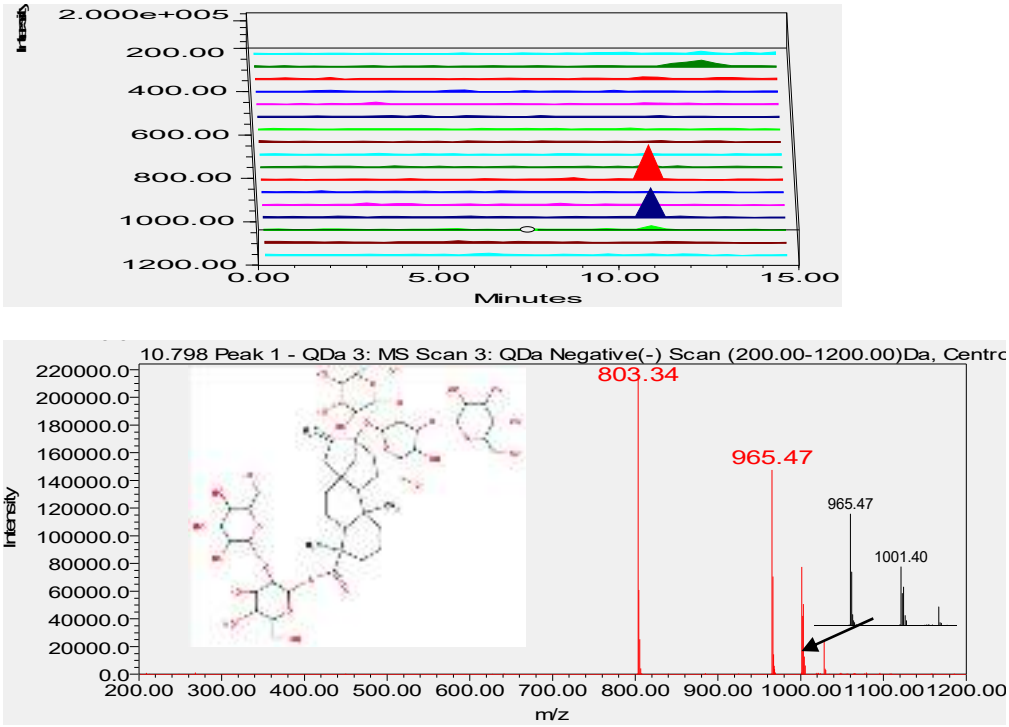


Fig. 7. UPLC-MS spectrum rebaudioside D

Substance 8-[M-H -] - m/z 1127, has been fixed on chromatograph -[M-H -] with m/z 803, 965. Retention time – 10.675 min, maximum absorption - UV- 211.9 nm. According to the base of METLIN compound masses, substance 8 corresponds to a steviol tri-glucoside-mono rhamnoside, ie rebaudioside D ($C_{50}H_{80}O_{28}$).

A molecule from the Rebaudioside D (M-H2O) - m / z 162) is a molecule that is m / z 787. As a result of [M-H+-glc] glucose cleavage ([M-H2O] - m / z 162) from rebaudioside D, one molecule remains - m / z 787, while 2 molecules remain after glucose cleavage [M-H+-2glc] - c m / z 625. Rebaudioside D loses 2 molecules of glucose and one molecule of rhamnose ([MH2O] - m / z 146), [M-H+-2glc - rham] and in negative mode m / z 479 is received. We get chlorine ion on the compound ([M-H^+ -Cl^-] - m/z 1127).

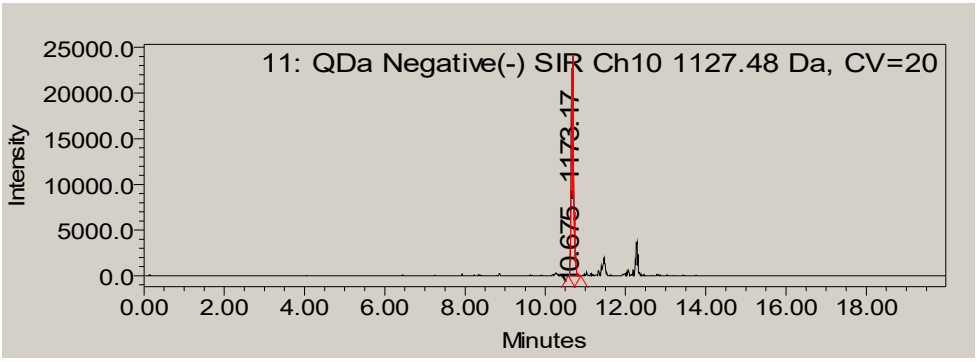


Fig. 8. UPLC-MS spectrum rebaudioside D

Substance 9-[M-H -] - m/z 949 is fixed on the chromatogram as a fragment - [M-H -] - m / z 787. Retention time - 11.880 min, maximum absorption - UV-211.9 nm. According to the base of METLIN compound masses, the substance 9 corresponds to a steviol tetra-glucoside-mono rhamnoside, ie rebaudioside C ($C_{44}H_{70}O_{23}$).

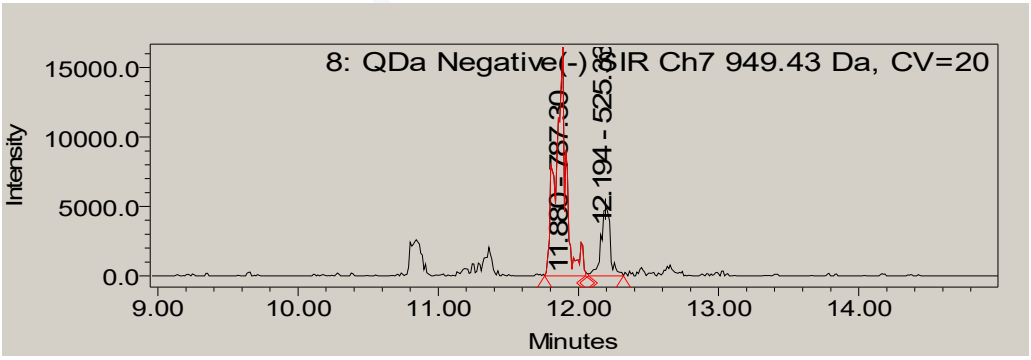
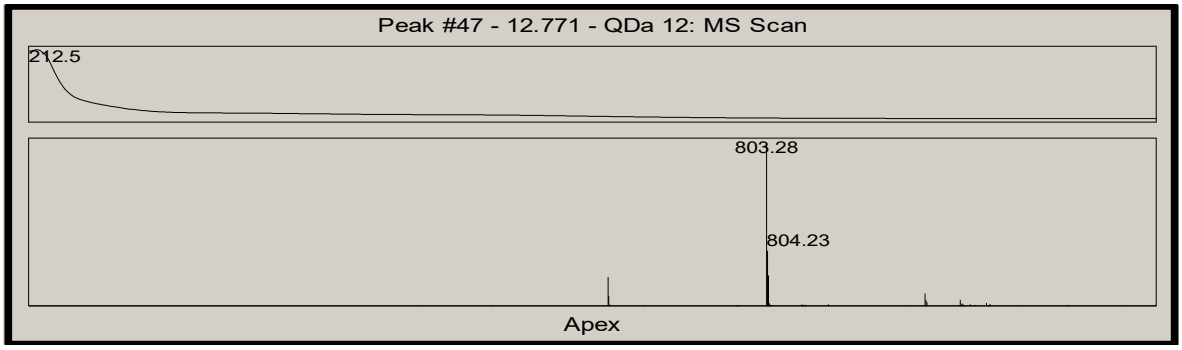


Fig. 9. UPLC-MS spectrum rebaudioside C

Substance 10-[M-H -] - m/z 965 is fixed on the chromatogram -[M-H -] with m/z 803, 1001. Retention time - 12.771 and 12.824 min, maximum absorption UV- 212.5nm. According to the base of METLIN compound masses, the substance 10 corresponds to a steviol tetra-glucoside, ie rebaudioside D ($C_{44}H_{70}O_{23}$).

The chromatograph has fixed 2 [M-H+] - m / z 965; fragmentation of compounds is used for their identification. Rebaudioside A is characterized by ([M-H+] - m / z 965) m / z 803 [M-H+-glc], while rebaudioside E ([M-H+]- m / z 965) is characterized by a fragment m / z 641, the result of 2 molecules cleavage from glucose is ([M-H+-2glc]-).



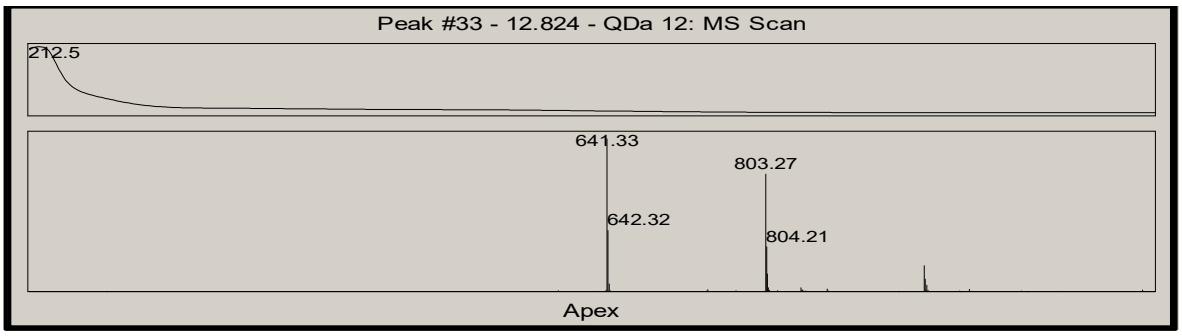


Fig. 10. UPLC-MS spectrum rebaudioside A

Substance 11-[M-H -] - m/z 935 is fixed on the chromatogram -[M-H -] as a fragment m/z 773.17, as it loses 1 molecule of glucose. Retention time - 11.787 min, maximum absorption UV- 211.9 nm. According to the base of METLIN compound masses, the substance 11 corresponds to a steviol tetra-glucoside, ie rebaudioside F($C_{43}H_{69}O_{23}$).

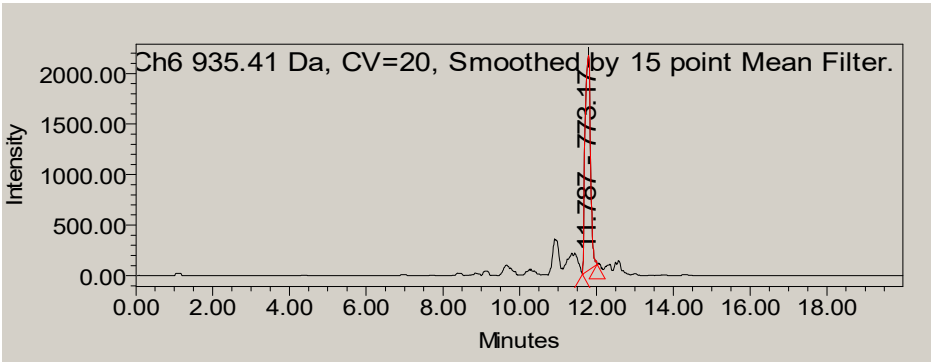


Fig. 11. UPLC-MS spectrum rebaudioside F

Substance 12-[M-H -] - m/z 787 is fixed on the chromatogram -[M-H -] with m/z 803, 965, as a product of their fragmentation. Retention time - 11.867 min, maximum absorption UV- 212.3 nm. According to the base of METLIN compound masses, the substance 12 corresponds to a steviol tri-glucoside, ie duglucoside A ($C_{38}H_{60}O_{17}$).

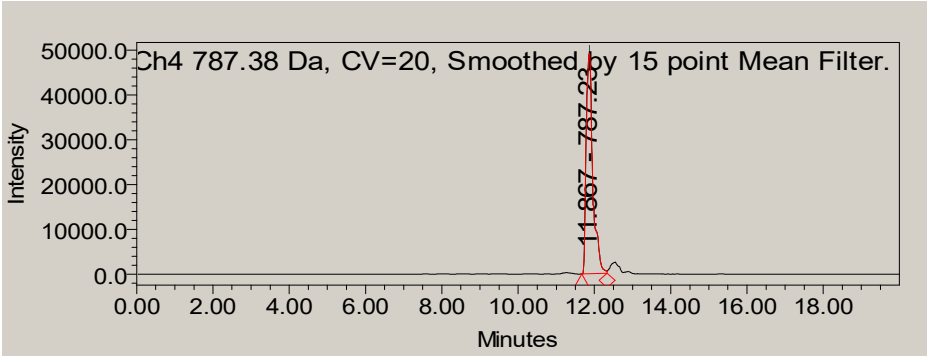


Fig. 12. UPLC-MS spectrum duglucoside A

Some compounds fragmentation ends with mm / z 479 and m / z 317 (steviol), resulting from glucose 3 and 4 respectively [M-H+-3glc] and [M-H+-4glc].

Table 1.UPLC-MS data of stevia terpene glycosides in negative ion mode from LC-ESI MS analysis

Comp.	Name	Molecule Formule	*m/z* $(M-H^+)^-$	*m/z* $(M-H^+)^+$
1	Steviol	$C_{20}H_{30}O_3$	317.24	319.21
2	Steviol -GLC		479.12	481.2
3	Steviol -2GLC [M-16]		625.13	627.12
4	Steviol -2GLC		641.33	643.21
5	Steviol -3GLC Deoxiglukoside [M-16]		787.17	789.13
6	Steviolbioside	$C_{32}H_{50}O_{13}$	641.34	643.31
7	Stevioside Steviol -3GLC	$C_{38}H_{60}O_{18}$	803.31	805.37
8	Rebaudioside A Steviol-4GLC	$C_{44}H_{70}O_{23}$	965.52	967.42
9	Rebaudioside C	$C_{44}H_{70}O_{23}$	949.46	951.42
10	Rebaudioside D	$C_{50}H_{80}O_{28}$	1127.47	1129.47
11	Rebaudioside F	$C_{43}H_{69}O_{23}$	935.41	967.4235
12	Dulcoside A	$C_{38}H_{60}O_{17}$	787.38	789.3758

CHAPTER 3. Study of Stevia leaf phenolic compounds with high performance liquid chromatography HPLC and UPLC method

The following phenolic compounds have been identified in the composition of Stevia leaf and its extract:

Substance 13-[M-H -] - m/z 353, the result of fragmentation is m / z is 191 peak. Retention time - 5.151 min, maximum absorption - UV-324.9 nm. According to the base of METLIN compound masses, the substance 13 corresponds to Chlorogenic acids Mono-caffeoylquinicacids (mono-CQA).

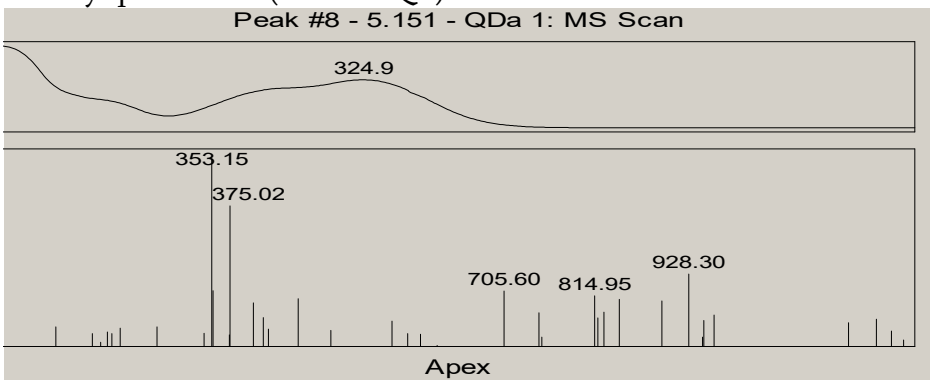


Fig. 13. UPLC-MS spectrum

Substance 14-[M-H -] - m/z 353, the result of fragmentation is m/z 191 and m/z 173 peaks. Retention time - 5.151 min, maximum absorption - UV-324.9 nm. According to the base of METLIN compound masses, the substance 14 corresponds to caffeoylquinicacids (CQAs).

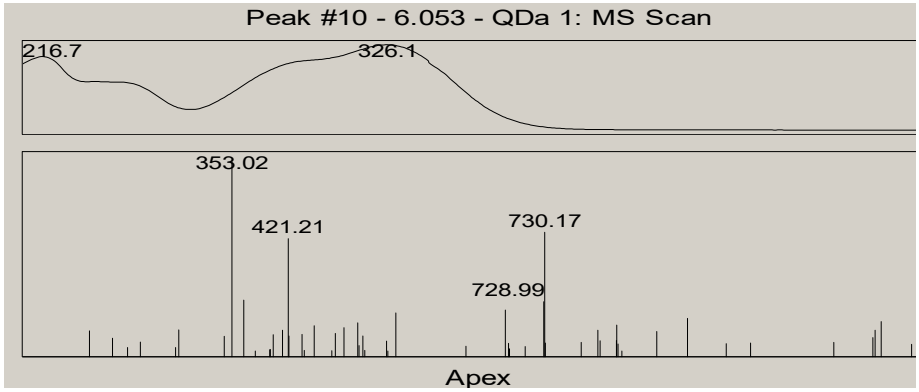


Fig. 14. UPLC-MS spectrum

Substance 15-[M-H -] - m/z 515, the result of fragmentation - m/z 353. Retention time - 5.151 min, maximum absorption - UV-324.9 nm. According to the base of METLIN compound masses, the substance 15 corresponds to 3,5-di-caffeoylquinic acid (3,5diCQA).

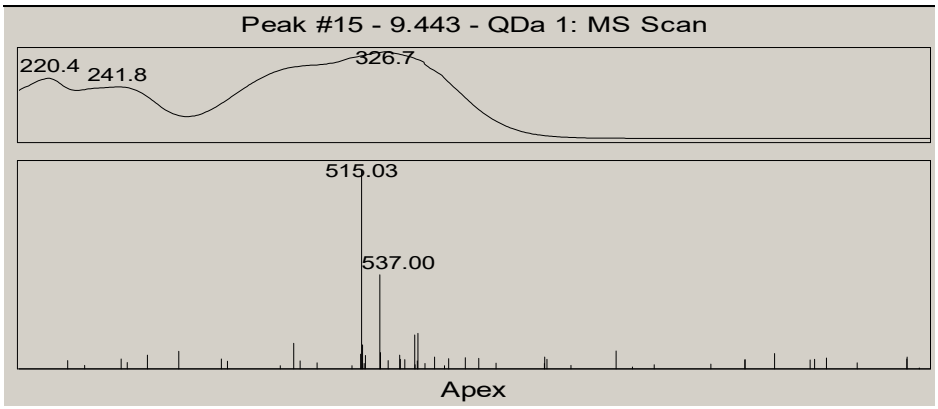


Fig. 15. UPLC-MS spectrum

Substance 16 -[M-H -] - m/z 515, the result of fragmentation is m/z 353. Retention time - 10.146 min, maximum absorption - UV-327.3 nm. According to the base of METLIN compound masses, the substance 16 corresponds to 4,5-dicaffeoylquinic acid (4,5diCQA).

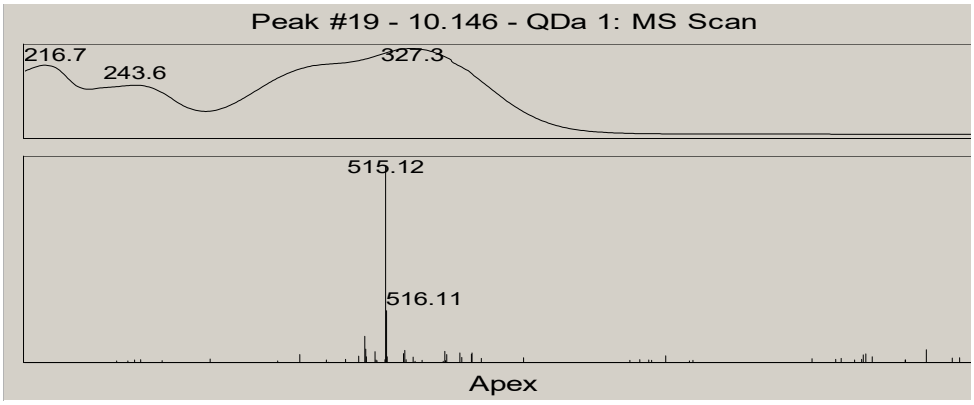


Fig. 16. UPLC-MS spectrum

Substance 17 -[M-H -] - m/z 463, the result of fragmentation is m/z 301. Retention time - 9.051 min, maximum absorption - UV-344 nm. According to the base of METLIN compound masses, the substance 17 corresponds to quercetin-galactoside.

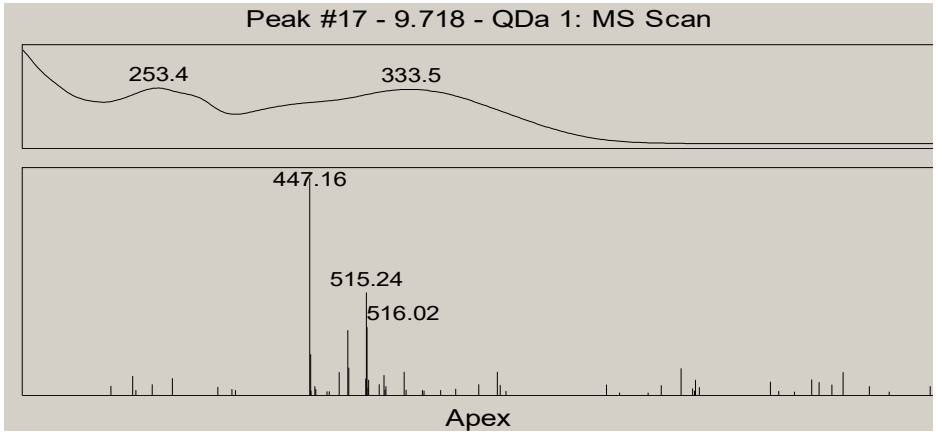


Fig. 17. UPLC-MS spectrum

Substance 18 - [M-H -] - m/z 609, the result of fragmentation is m/z 301. Retention time - 9.051 min, maximum absorption - UV-344 nm. According to the base of standard compounds and METLIN compounds masses, as well as compared to the standard compound, the substance 17 corresponds to Rutin.

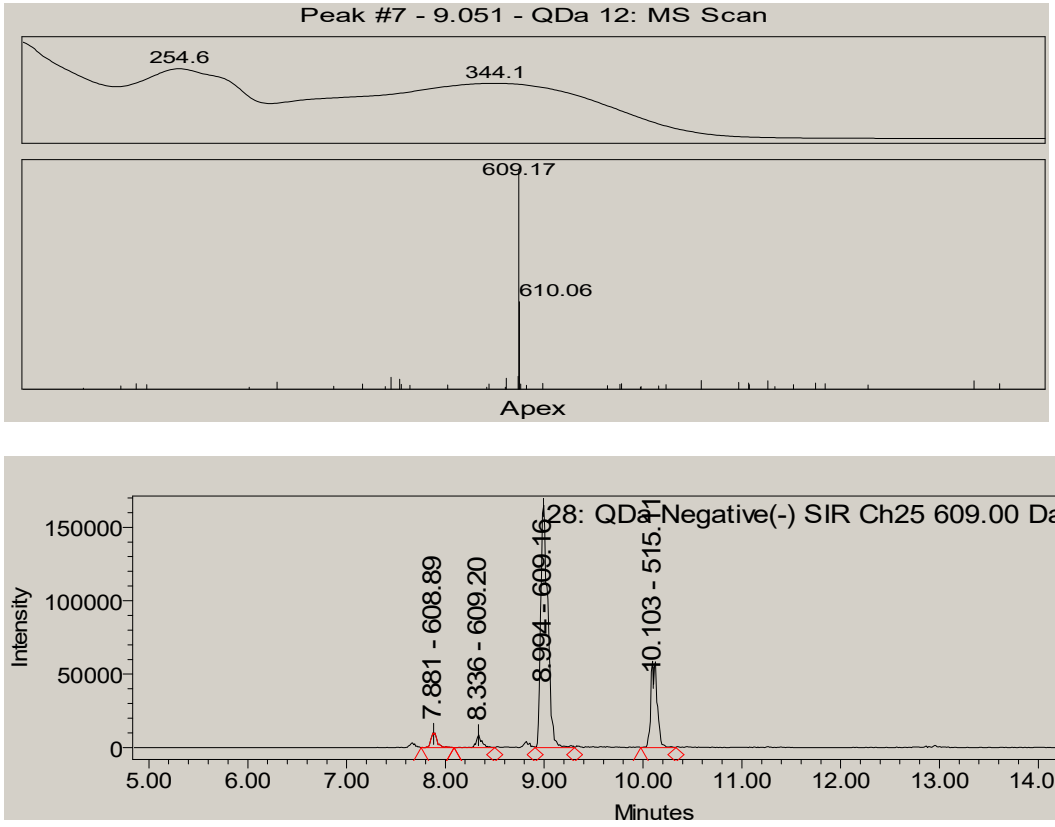


Fig. 18. UPLC-MS spectrum

Substance 19-[M-H -] - m/z 447, the result of fragmentation is m/z 301. Retention time - 9.955 (in MS, PDA) min, maximum absorption - UV-360 nm. According to the base of standard compounds and METLIN compounds masses, the substance 19 corresponds to quercetin-rhamnoside.

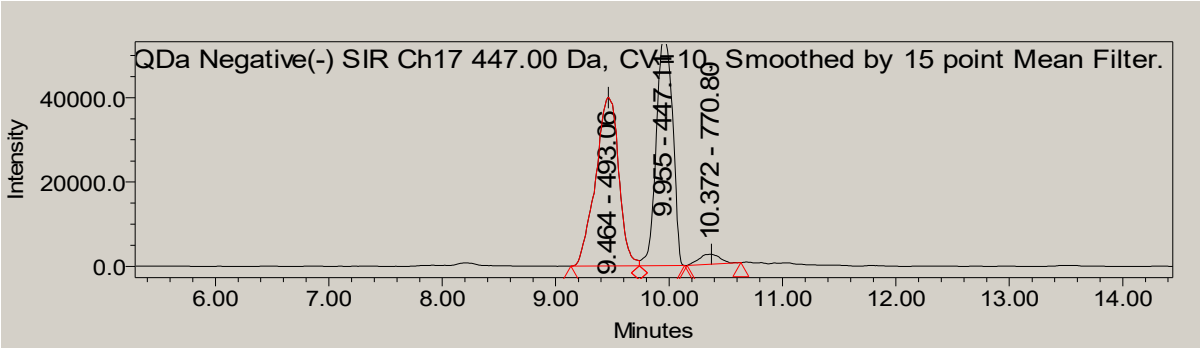


Fig. 19. UPLC-MS spectrum

Substance 20-[M-H -] - m/z 433, the result of fragmentation is m/z 301. Retention time - 9.605 min, maximum absorption - UV-360 nm. According to the base of standard compounds and METLIN compounds masses, the substance 20 corresponds to quercetin- pentoside.

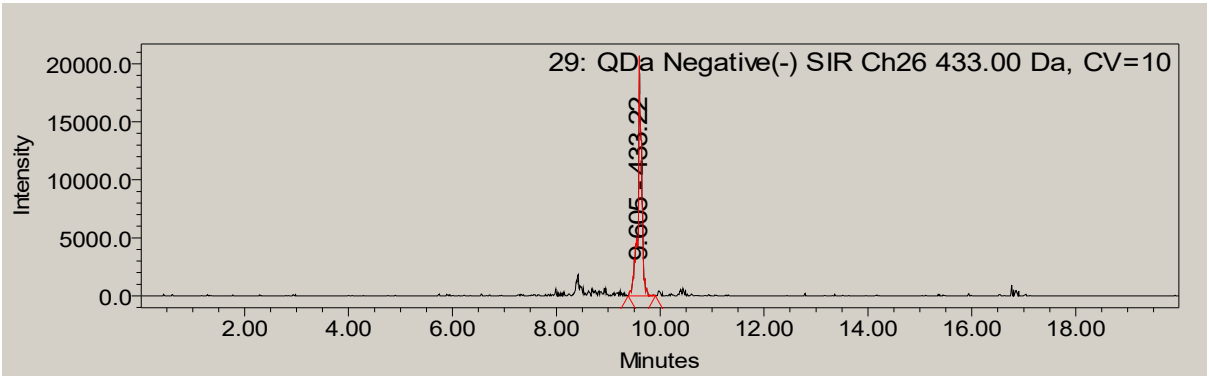


Fig. 20. UPLC-MS spectrum

Table 2.UPLC-MS data of stevia phenolic compounds in negative ion mode from LC-ESI MS analysis

#	Compound	Molecular Formula	m/z [M-H +] –
1	3-caffeoylquinic acid (3CQA)	C16H17O9	353
2	4-caffeoylquinic acid (4CQA)	C16H17O9	353
3	Rutin	C27H30O16	609
4	Quercetin-rhamnoside	$C_{21}H_{20}O_{11}$	447
5	Quercetin-galactoside		463
6	3,5-di-caffeoylquinic acid (3,5diCQA)	C25H24O12	515
7	4,5-dicaffeoylquinic acid(4,5diCQA)	C25H24O12	515

CHAPTER 4. The content of steviol glycosides in various raw materials and the preparations obtained from them

A quantitative study of steviol glycosides has been performed on the basis of calibration curves, constructed using standard stevioside and rebaudioside. Individually extracted ionic SIR 803 (rebaudioside) and SIR 641 (stevioside) chromatographic characteristics have been used for the curve construction. Calculations of other sweet glycosides were made with respect to stevioside.

For the quantitative analysis of sweet diterpene glycosides, the acquired varieties were compared with the local (spontaneous) forms. In Stevia leaves, stevioside is the dominant in all cases: America № 3 - 11.03%, America № 4 - 12.34%, local spontaneous variety side leaf - 10.24%, main spontaneous leaf - 11.41%. By quantitative indicators, stevioside prevails over the content of other glycosides. In any case, their content is up to 55-60% of the total content of sweet diterpene glycosides. The content of rebaudioside A, respectively, is relatively less (30-38%), the rest are minor compounds, among which there is a relatively large amount of rebaudioside C (from 5.4 to 8.2%). It is noteworthy that the central leaves of the plant, compared with the side leaves, contain a a relatively small amount of sweet compounds (10.2 g / 100 g and 11.4 g / 100 g, respectively).

Table 3.The content of sweet diterpene glycosides in the leaves of Stevia (per mg / 100 g dry weight)

Sampler	m/z^- 641	m/z^- 803	m/z^- 935	m/z^- 949	m/z^- 1127	total
America 3	7304	3686	269	747	330	12336
America 4	6359	3357	256	712	342	11026
Local side	4760	3924	269	849	434	10236
Local central	6208	3896	218	615	472	11409

Table 4. The content of sweet diterpene glycosides in the extracts of Stevia leaves (mg/l)

Sampler	m/z^- 641	m/z^- 803	total
initial extract	3819	3016	6935
Filtrate 2000 Dal	4217	3062	7279
Concentrate 2000 Dal	3764	2927	6691
Filtrate 1000 Dal	4540	3030	7570
Concentrate 1000 Dal	3660	2823	6483

Table 5.The content of sweet diterpene glycosides in SFE extracts of Stevia leaves (mg / 50 ml)

Sampler/fraction	m/z^- 641	m/z^- 803	Total
SFE 20	31.77	37.36	69.13
SFE 21	38.29	37.7	75.99
SFE 22	32.45	37.72	70.17
SFE 23	26.41	31.21	57.62
SFE 24	25.72	33.27	58.99
SFE 25	23.35	29.02	52.37
SFE 26	19.05	25.63	44.68
SFE 27	17.39	24.92	42.31
SFE 28	24.25	28.95	53.20

SFE 29	28.56	30.86	59.42

Some changes were observed during the refining of the Stevia leaf extract. Distillation of the extract in the filter 2000 Dal leads to an increase in sweet diterpene glycosides; respectively, their content decreases in the concentrate (from 6935 mg / l to 6691 mg / l), and increases in the filtrate (7279 mg / l). The corresponding changes occur in the pore filter 1000 Dal: in the filtrate it increases (7570 mg / l), and in the concentrate it decreases (6483 mg / l). Due to the modification of membrane pores, it becomes possible to refine the extract of Stevia.
SFE extraction of Stevia leaves into cosolvent, using ethanol, allows to obtain preparations with a high content of stevioside and rebaudioside (76 mg / 50 ml).

CHAPTER 5. Stevia Lipid Analysis

10 g of a dry leaf of Stevia was extracted with chloroform in a Soxhlet apparatus until complete removal of pigments and other lipid compounds (8 hours). The solvent was evaporated under vacuum and the total amount of lipids was measured gravimetrically.
For methyl ester, the lipid extract was dissolved in 10 ml of chloroform. The obtained from the extract 1 ml of the substance was added by 200 μl of methyl potassium hydroxide solution (2 Molars) and 1 g of sodium hydrofluct monohydrate (NaHSO4), which resulted in the GC-analysis of the obtained Stevia methyl esters of lipids.
The studied sample was filtered from mechanical impurities. 1 ml of the filtered sample was transferred to a centrifuge tube by adding 0.5 ml of 2 normal 96% KOH alcohol (ethanol can be used). Then 10 ml of hexane (total volume 11.5 ml) was added, agitated until complete dissolution (at least 30 seconds) and centrifuged for 10 minutes at 1000 revolutions / min. Then from the upper organic fraction of the sample was taken 1 μl and injected into the chromatograph with an injector. Chromatography was performed with a temperature gradient in three stages. In particular, chromatography began at 140 ° C and lasted 4 minutes. In the second stage, at a speed of 20 ° C / min, the temperature increased to 220 ° C and the chromatography was carried out for 16 minutes. At the third stage, at a speed of 7 ° C / min and a temperature of up to 300 ° C, chromatography continued for 7 minutes. The total chromatography time was 42.43 minutes. The quantitative content of carbon dioxide was determined by the peak area in percent with an accuracy of 0.01%.

Identification of components obtained, using chromatographs, was carried out by comparison with the data of a sample of known content, as well as on the basis of literature data. The results of the analysis are shown in Table 1.

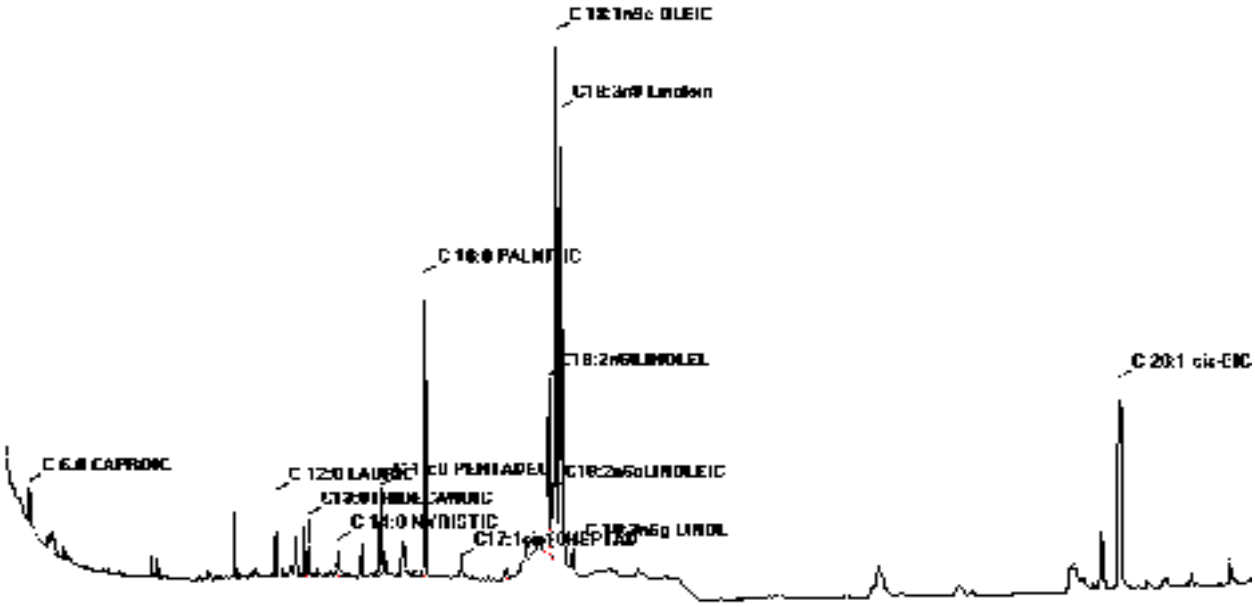


Fig. 21 GC Chromatogram stevia lypides

Table №6. Component composition of carbonic acid

Peak	Component Name	RT (min)	Area %
1	C 6:0 CAPROIC	3.373	0.069
2	C11:0 UNDECANO	9.540	0.018
3	C13-1	11.307	0.338
4	C13:0TRIDECANOIC	11.390	0.337
5	C 14:1 MYRISTOL	11.968	0.152
6	C 14:0 MYRISTIC	12.228	0.025
7	C 15:0 PENTADEC	13.317	0.118
8	C 16-2	13.643	0.792
9	C 16:1 PALMITOLE	14.243	9.339
10	C 16:0 PALMITIC	14.558	0.535
11	C17:1cis10HEPTAD	15.408	10.535
12	C 17:0 HEPTADEC	15.623	0.099
13	C 18:3n6g LINOLENIC	17.575	6.065
14	C18:2n6cLINOLEIC	17.733	6.571
15	C 18:1n9c OLEIC	18.137	37.425
16	C18:1n9t Elaidic	18.318	0.362
17	C18:0 STEARIC	18.520	1.743
18	EicosatriC20:3	19.573	7.266
19	C 20:0 ARACHIDIC	19.772	12.801
20	C 21:0 Heneicosano	20.508	2.357

Chromatographic research has demonstrated that the oil, obtained from Stevia leaves, contains C 16, C17, C18 and C20 with dominant C 18 carboxylic acid, which contains 37,425% of total fat content.

CHAPTER 6. Study of antioxidant activity of Stevia leaf and its derivative products using DPPH method

Stevia rebaudiana extract has antioxidant activity, which is caused by a complex of phenolic compounds. Among the different varieties of introduced plants, the most active one is an extract obtained from the leaves of plants introduced from Paraguay, 0.341 mg of which can produce 0.01 mM DPPH 50% inhibition. Local spontaneous populations are less active. The second extract (20% alcohol extract) is almost in all cases active and can inhibit about 0.1 mg. The antioxidant activity of Stevia significantly varies during processing. The main difference is based on the level of extract refining. A preparation, which is 100 times sweeter than sucrose, is especially active (only 0.015 mg can be inhibited); this indicator is reduced by almost 7 times in the preparation which is 200 times sweeter (0.107 mg), and a completely refined preparation (300 times sweeter) significantly (45 times) loses its antioxidant activity.

Table No 7. Antioxidant activity of Stevia leaves and products

Sampler name	IC50 mg of sample	Antioxidant activity
# 3.Stevia Rebaudiana Bertoni, South America I extract	0.364	27
#4.Stevia Rebaudiana Bertoni, from Paraguay I extract	0.341	27
Stevia of Introduced in Georgia outer leaves I extract	0.460	40
Stevia of Introduced in Georgia internal leaves I extract	0.454	40
# 3.Stevia Rebaudiana Bertoni, South America II extract	0.102	12
#4.Stevia Rebaudiana Bertoni, from Paraguay II extract	0.100	14
Stevia of Introduced in Georgia outer leaves II extract	0.116	27
Stevia of Introduced in Georgia internal leaves II extract	0.115	28
Stevia powder Sweeter than 100 times sugar	0.015	442
Stevia powder Sweeter than 200 times sugar	0.107	91
Stevia powder Sweeter than 300 times sugar	0.698	17
Filtrate 2000 dalton in membrane filter	0.045	134
Filtrate 1000 dalton in membrane filter	0.085	87
Concentrate 1000 dalton in membrane filter	0.075	77

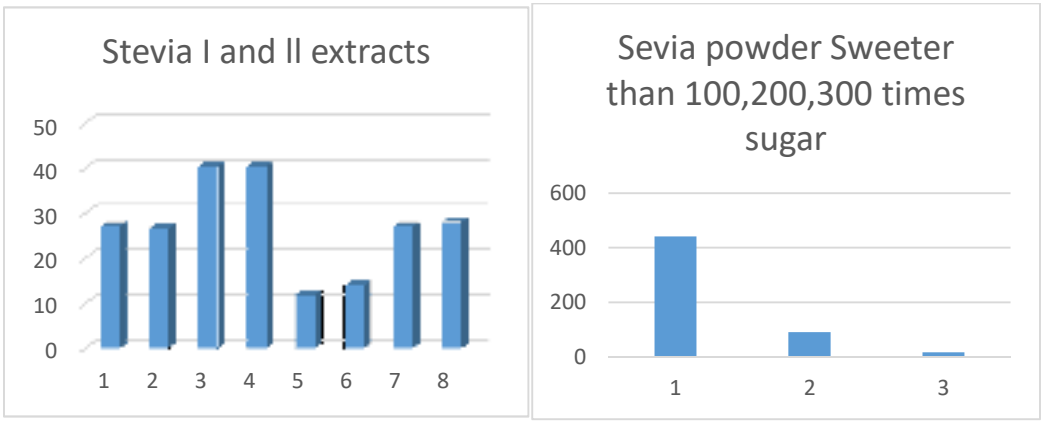
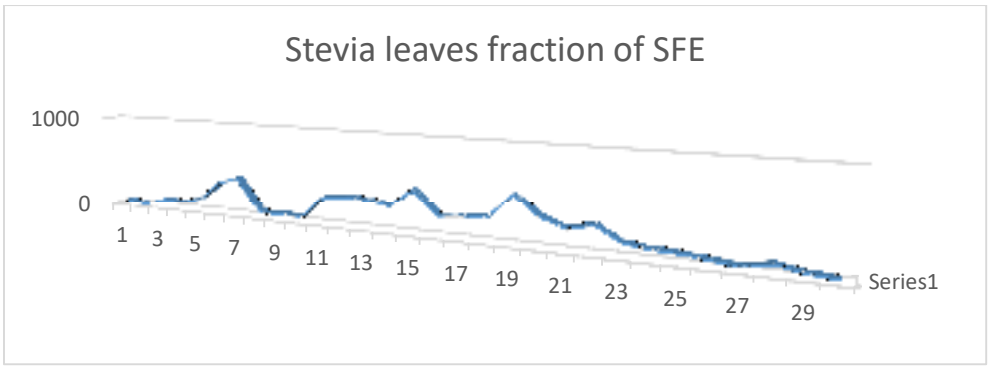


Fig. 21. The percent inhibition of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical with different extracts of Stevia leaves



Stevia leaves are becoming more promising, not only due to the use in the production of virtually calorie-free sweeteners, but also because of its high antioxidant activity.

CHAPTER 6. **Stevia leaf SFE** (Supercritical Fluide Extraction)

The processing of Stevia's pre-dried leaf was carried out by fluid (inert gases-carbon dioxide and co-solvent-ethanol) extraction of supercritical pressure (Waters SFE -100-2-C10).

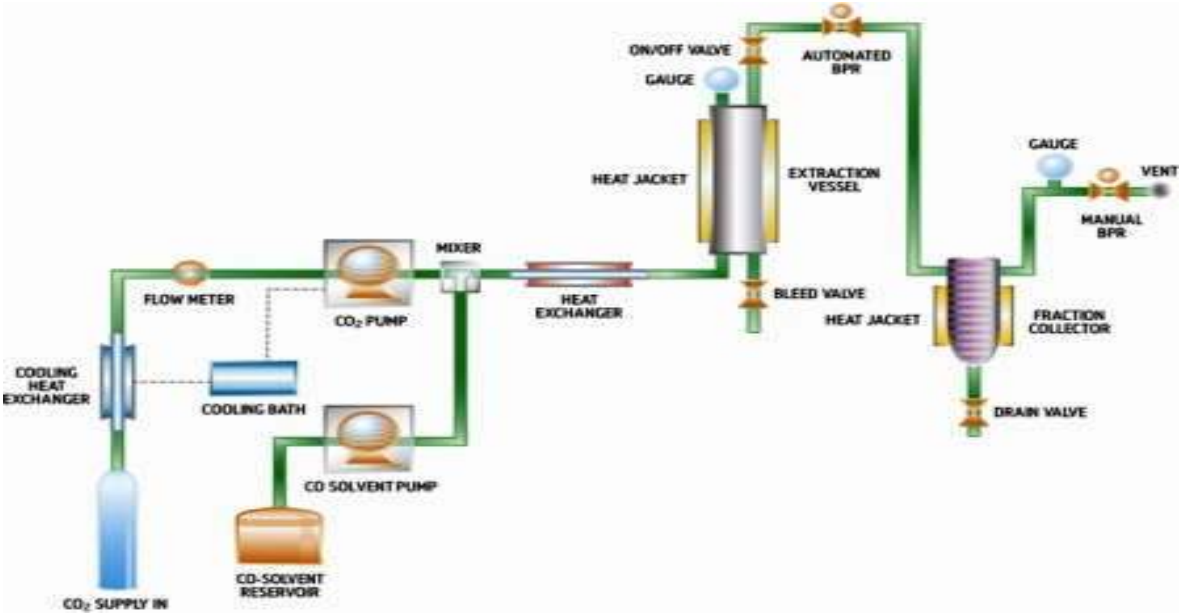


Fig. 22. General view of SFE 500 equipment.

The apparatus consists of the following main components: carbon dioxide reservoir, solvent (pH) pump, co-solvent pump, co-solvent reservoir, mixer, extractor, evaporator-cyclone and other controlling equipment which is managed by a computer.

Supercritical Fluid Extraction (SFE) of Stevia leaf. There have been selected two methods of Fluid Extraction. The first extraction method was used to produce diterphenoidal glycosides from Stevia leaves, while the purpose of the second method is the removal of the obstructive substances (including colored ones) from the Stevia leaves, what allows us to obtain the total preparation of diterphenoidal glycosides by hot extraction of leaves (ethyl alcohol / water mixture). 31 fractions have been obtained from 10 grams of green dried Stevia leaf, extracted by the SFE method.

The mode of conducting the SFE method consisted of four stages; the following fractions were obtained:

First stage - extraction by carbonate dioxide of supercritical pressure;

Fraction 1 -30 min, 500 bar at 40°C at speed of carbon dioxide 20 g / min;

Fraction 2 - 20 min, 500 bar at 60°C at speed of carbon dioxide 20 g / min;

Fraction 3 - 20 min, 500 bar at 80°C at speed of carbon dioxide 20 g / min;

At the second stage, there was added 5% co-solvent (96% ethanol)

Fractions 4-7 - 350 bar at 60°C at speed of carbon dioxide 20 g / min;

Third stage -10% co-solvent was added (96% ethanol)

Fractions 8-16 - 350 bar at 60°C at speed of carbon dioxide 20 g / min;

Fourth stage - 5% co-solvent was added (50% ethanol/water)

Fractions 16-24 - 350 bar at 60°C at speed of carbon dioxide 20 g / min;

Fifth stage - 5% co-solvent was added (96% ethanol/water);

Fractions 24-31 - 350 bar at 60°C at speed of carbon dioxide 20 g / min.

The equipment is depicted in Fig. 23, while the first stage is graphically illustrated in Fig. 2,3

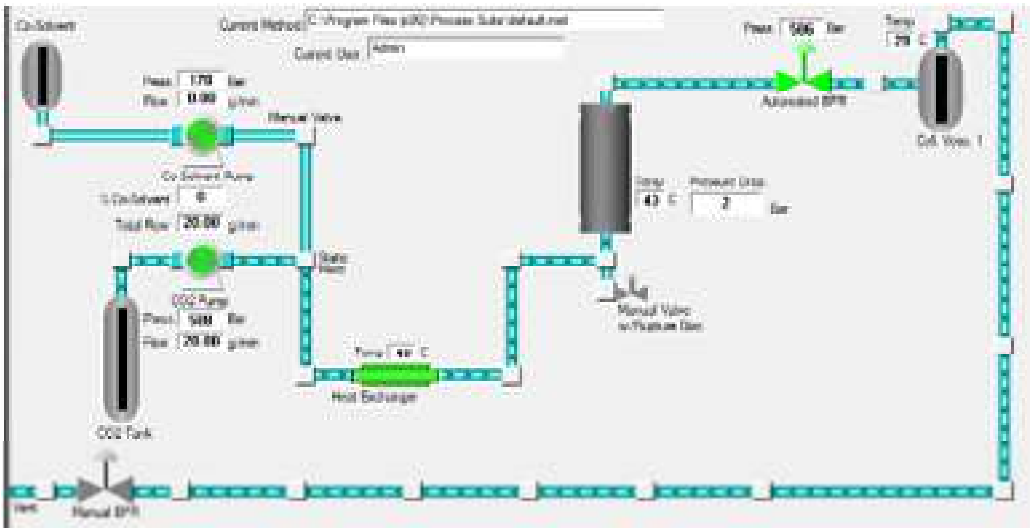


Fig. 23 Processing Scheme

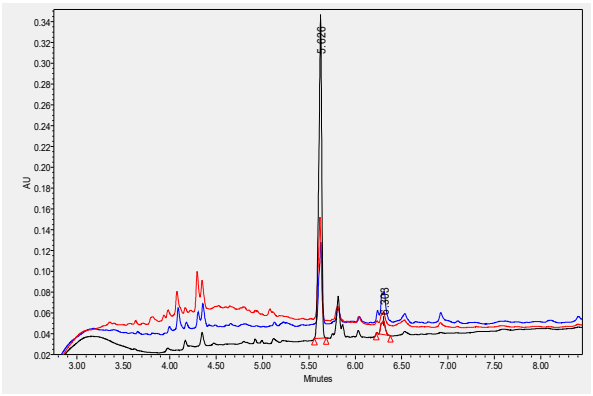
Fig 24. SFE Praparat in the recycler

At the first stage of Stevia leaf processing through SFE method, soluble lipophilic compounds were extracted in organic solvents. Therefore, naturally, chlorophyll A and B (37.54-1.96 mg / g, 24.16-0.427 mg / g respectively) and carotene 19.1-1.0 mg / g prevail in fractions 1-8. The extraction of phenol carbonate acids, catechins and flavonoids (1-8 Fractions -150-7.6 mg / c respectively) was carried out at the water flow in the leaf.

There are almost no pigments in fraction 8. The amount of pigments after the addition of co-solvent to the fractions (31- 9) is in the form of a trace, while in fractions 17- 19 it exceeds 3 mg / g. The number of all phenolic compounds increases from fraction 17. The exception is phenol carbonate acid, which content varies without any regularity. This can be explained by various compounds of organic solvents in different solutions of phenol carbonate acid. The extract is rich in dry substances in fractions 1-8. It varies from 7.75% to 0.27%. Their content reduces in fractions 9 - 15 and, therefore, the number of analyzed substances also decreases. The content of extracted substances, as well as catechins, phenol carbonate acids and common flavonoids increases in fractions 16-20. The composition of extracted compounds gradually decreases in fractions 21-31, while the total number of individual components of phenolic natural compounds is preserved.
The sweet terpenoidal glycoside was obtained from the analyzed fractions 20-29 (total amount of steviosides and rebaudiosides 12000- 7000 ppm respectively)(Fig. 5). However, its amount is 500 ppm in fraction 31. As a final product, two preparations (sweeter than sugar in 100 times and 300 times respectively) were obtained. The total amount of steviosides and rebaudiosides in them was 29% and 93 % respectively.

The total glycosides have been identified and quantified in the same fractions (20,29) and the both preparations by UPLC-PDA method.

The antioxidant activity of the obtained fractions and preparations was determined, which increases with the total growth of phenolic compounds of different types (fractions 8-23) (Table 8).



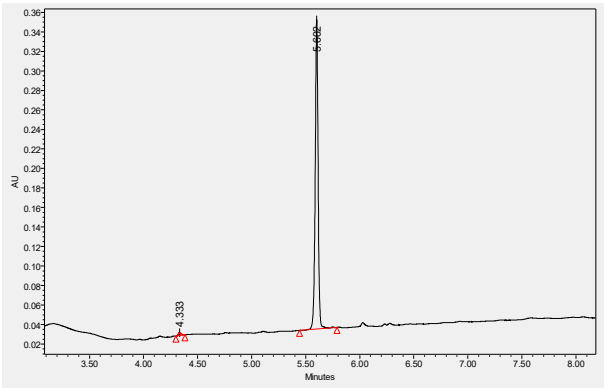


Fig. 25. stevia 100 UPLC-PDA -214 nm Fig. 26 Rebaudioside A UPLC-PDA -214 nm

Fig. 27stevia 300 UPLC-PDA -214 nm

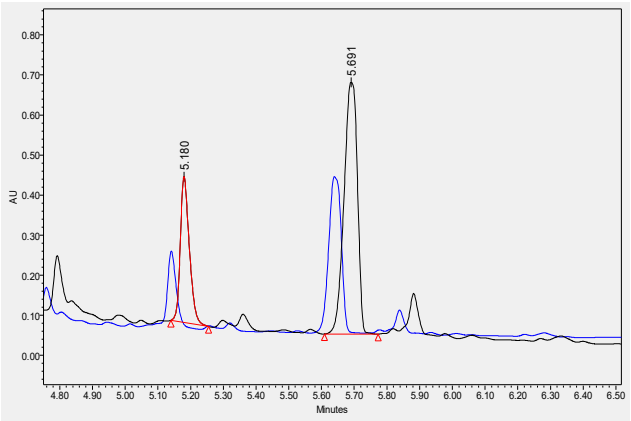


Fig. 28. stevia fraction 20, 29 UPLC-PDA -214 nm

Table 8 Sweet diterpene glycoside in SFE fractions

	Name	Retention Time	Area	% Area	Height	Amount	Units
1	rebaudioside A +stevioside	5.602	594316	99.04	317392	2000	ppm
2	Fraction 29	5.640	1052679	76.29	388070	7084,98	ppm
3	Fraction 20	5.640	1789435	71.92	388070	12043,66	ppm
4	Stavia 100	5.619	225691	77.26	98311	29,00	%
5	Stevia 300	5.626	688461	94.38	306916	93,64	%

Using the SFE method, we have fractionated the components of Stevia leaf and identified chlorophyll A and B, common carotenes, common flavonoids, catechins and phenol carbonate acids for each fraction. Two preparations, containing different quality of sweetness, have been obtained; the quantitative content of glycosides in them was determined as well. The antioxidant activity has been established both for fractions and preparations.

CHAPTER 7. Study of Stevia leaves cations with a chromatograph using a conductometric detector

Some of the cations of Stevia leaf and its preparations have been analyzed by the chromatographic method, using conductometric detector.

Standards lithium hydroxide monohydrate (Li +), sodium chloride (Na +), ammonium chloride (NH +), potassium chloride (K +), magnesium hydrate (Mg2 +), calcium nitrate tetrahydrate (Ca2 +), strontium nitrate tetrahydrate (dihydrate sodium barium + sodium (Sr2 +), barium chloride dihydrate (Ba2 +) (FisherScientific, EDTA (Serva). Isocratic HPLC pump-Waters 1515), IC-PakCationMD chromatographic column, eluent 3 mM HNO3 / 0.1 mm EDTA, eluent conductivity 1250 ± 50 μs, basic sensitivity 2000 μs, integrator sensitivity 0.01 μs, column temperature 350 ° C, polarity-negative. The total amount of the main cations of stevia leaves is about 5%. The preparation obtained from stevia naturally contains water-soluble cations, which are 100 times higher than the sugar content and make up a little more than 5%. Subsequent refining of the drug in the first stage causes a sharp increase in the number of cations; in the preparation, which is 200 times sweeter than sugar, there are more than 8%. And in the preparation of Stevia, which is 300 times sweeter than sugar, the total cation content is up to 0.3%.

The content of cations in stevia leaves and preparations

Table 9

Amaunt PPM	Na+	NH4+	K+	Mg2+	Ca2+	total mass %
Stevia central leaves	895,2	2431,6	46139,6	1276,4	1485,5	5,227
Stevia Side leaves	928,0	2441,6	42795,1	1845,0	2520,5	5,059
Stevia America 4	447,8	158,9	23169,0	675,2	612,2	2,506
Stevia America 3	579,4	784,3	25914,7	1961,6	2371,5	3,161
Stevia 100	1261,6	1414,9	39163,7	4413,7	5189,4	5,147
Stevia 200	967,8	3413,7	75024,5	939,4	234,5	8,058
Stevia 300	1335,1	126,8	1210,3	0,000	382,0	0,305

Stevia leaf collects potassium ions in particularly large quantities. Their content is 80% more than that of all cations. It is interesting that at a certain stage of purification of the drug, there can be observed the concentration of potassium ions. The resulting preparation is of particular interest because of the high content of potassium.

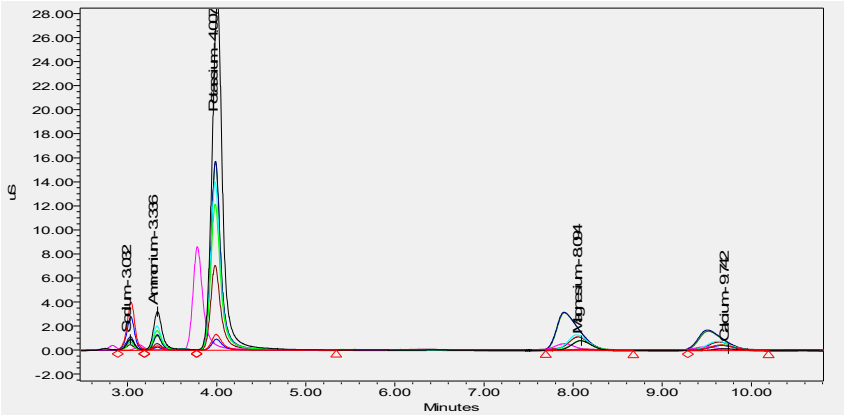


Fig. 29 Chromatograms of Stevia leaves and preparations

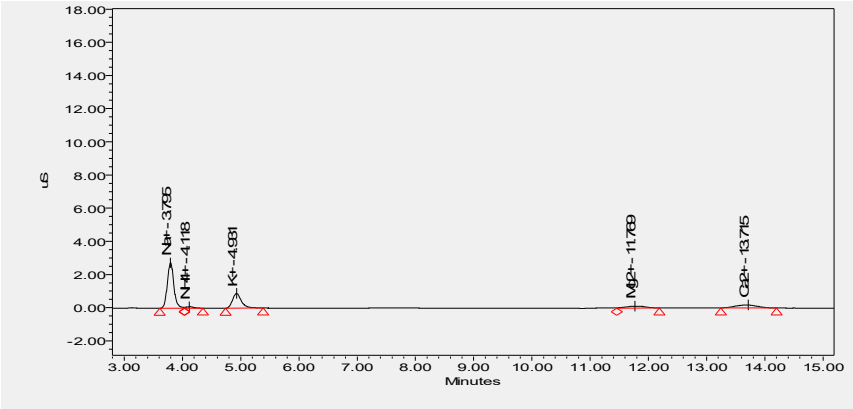


Fig. 30 Chromatogram of the drug, which is 300 times sweeter than sugar

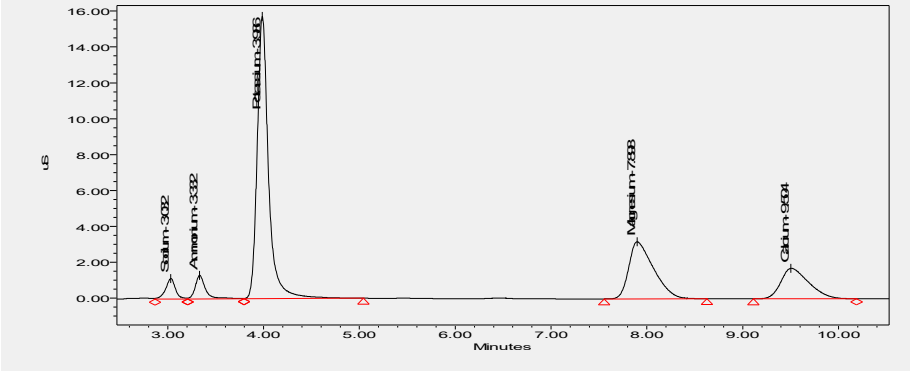


Fig. 31 Chromatogram of the drug, which is 100 times sweeter than sugar

CHAPTER 8. Study of stevia leaf essential oils using gas chromatography

The study of Stevia leaf essential oils, obtained by hydrodistillation, was carried out using a gas chromatograph (TRACE ™ 1310 Gas Chromatograph - Thermo Scientific) on a SGE BPX5 Capillary GC Column chromatographic capillary column 30 m long, 0.25 mm in diameter and with a stationary phase particle size of 0.25 μm. The stationary phase was represented by 5% Phenyl Polysilphenylene-siloxane.

During chromatography a mobile phase is represented by helium, which speed of movement is 0,700 ml / min. The research sample was injected through the SGE Analytical Science using a 10 μl microsyringe.

The ratio of sample injected into the column to helium emission in the stream was 1/100. Chromatography was carried out at a temperature gradient in four stages. In particular, the chromatography was started at a temperature of 50 ° C and brought to 250 ° C at a speed of 3 ° C / min (second stage); the chromatography lasted 10 minutes. At the third stage, at a speed of 10 ° C / min and a temperature that increased to 270 ° C, chromatography was continued for 3 minutes. At the fourth stage, at a speed of 21.4 (° C / min), the temperature reached 320 ° C and lasted for 5 minutes. The whole chromatographic implementation time was 89.0 minutes. The essential oils recovered by chromatography were detected on an alu-ionization detector. The quantitative content of essential oils was determined with an accuracy of up to 0.01% in percentage according to the peak area.

Aromatic foliage complex of Stevia was obtained by hydrodistillation. 100 g of dried leaf (crushed) together with 3 liters of water was placed in a flask. Distillation was carried out using a Cleverger-type apparatus (Fig. 6) for 3 hours. Condensation occurred in a refrigerator at temperature - 0.0 ° C. The obtained essential oil was extracted with hexane, 0.5 µl of the organic part of which was centrifuged (2 minutes at 1350 revolutions / min) injected on the chromatograph.

The identification of the components obtained by chromatography was carried out by comparing sample data of a well-known content; the specific terpenoid composition of Stevia essential oils was established. The results of the analysis are given in chromatogram No. 1.

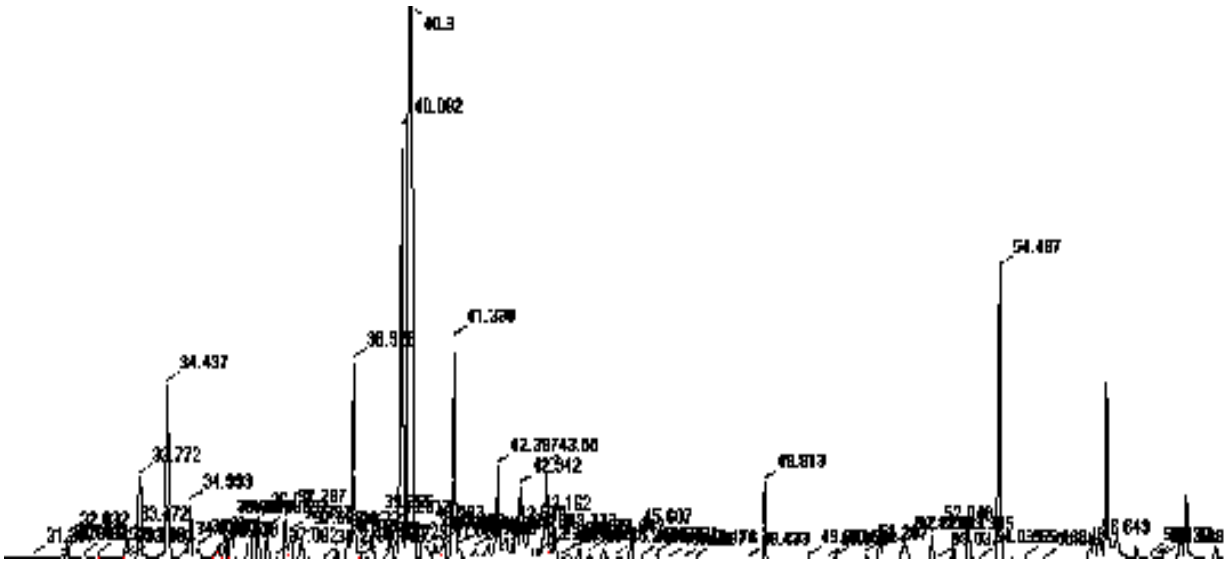


Fig. 32 Essential oils Chromatogram

Table №10. Component composition of essential oils.

Peak Number	Component Name	Peak Time, (min)	Area, %	Peak Number	Component Name	Peak Time, (min)	Area, %
1	a-Thujene	8.753	0.056	34	Peak 21	39.032	4.085
2	Peak 1	9.643	0.052	35	Peak 22	39.462	0.831
3	a-Pinene	11.570	0.424	36	Peak 23	39.887	0.742
4	Peak 2	13.588	2.613	37	Silphinene	40.163	11.685
5	y-Terpinene	13.970	0.043	38	a-Longipinene	40.500	30.730

6	Nonanal	15.812	0.210	39	Peak 24	40.815	0.068
7	1, 8-eucalypto	16.022	0.032	40	Peak 25	40.965	0.386
8	Peak 3	19.073	0.528	41	Peak 26	41.210	0.495
9	Peak 4	21.372	0.077	42	Modheph-2-ene	41.633	5.757
10	Peak 5	22.442	0.056	43	Peak 27	41.637	0.218
11	Perilaldehyde	23.815	0.108	44	Peak 28	42.282	0.592
12	Undecanal	26.107	0.100	45	a-Isocomene	42.487	2.366
13	2.6-Dodecadien	32.122	0.394	46	Peak 29	42.703	0.418
14	a-Humulene	32.518	0.071	47	Peak 30	42.857	0.361
15	a-Sellnene	33.575	0.502	48	Peak 31	43.267	1.003
16	Peak 6	33.863	1.763	49	Z-Caryophyllen	43.390	0.046
17	Thymol methyl	34.552	3.891	50	Peak 32	44.480	0.290
18	(E,E)-a-Farnes	34.927	0.271	51	Peak 33	44.688	0.142
19	Peak 7	35.087	1.326	52	Peak 34	45.122	0.349
20	b-Cadinene	35.290	0.317	53	Peak 35	45.713	0.570
21	Peak 8	35.900	0.680	54	E-Caryophyllen	48.917	1.694
22	Peak 9	36.105	1.077	55	Peak 36	50.605	0.081
23	Peak 10	36.497	0.782	56	Peak 37	51.377	0.149
24	Peak 11	36.727	0.937	57	Peak 38	52.950	0.663
25	Peak 12	36.950	0.766	58	Peak 39	53.465	0.425
26	Peak 13	37.355	1.002	59	Peak 40	54.615	7.197
27	Peak 14	37.495	0.631	60	Peak 41	55.982	0.039
28	Peak 15	37.778	0.538	61	Peak 42	57.553	0.154
29	Peak 16	37.923	0.063	62	Peak 43	58.212	0.130
30	Peak 17	38.138	0.036	63	Peak 44	63.865	0.188
31	Peak 18	38.268	0.061	64	Peak 45	65.250	0.158
32	Peak 19	38.638	0.361	65	Peak 46	67.870	0.186
33	Peak 20	38.828	0.158				

As a result of our chromatographic research, 65 components have been found in the essential oils of Stevia leaves. Among them there were identified 19 components, six of which are dominant. In particular:

Thymol methyl(3.891%), Silphinene(11.685%), a-Longipinene(30.730%), Modheph-2-ene(5.757%), a-Isocomene(2.366%), E-Caryophyllen(1.694%), a-Thujene, a-Pinene, y-Terpinene, Nonanal, 1,8-eucalyptol, Perilaldehyde, Undecanal, 2.6-Dodecadien, a-Humulene, a-Sellnene, (E,E)-a-Farnes, b-Cadinene, Z-Caryophyllen,

CHAPTER 9. Research of the Stevia Infrared spectrum

We have examined from 700 to 4000 nm of the infrared spectrum of Stevia. During the research there was used equipment *Cary 630 FTIR* of *Agilent* company. The study revealed the maximum amount of absorption. Namely: 3377.0-3388.2 cm^{-1}, which corresponds to the group; 2927.8-2937.1 cm^{-1}, which corresponds to -CH and alcohol group OH; 1654.9-1735.1 cm^{-1}, which corresponds to the group C = O; 1600.9-1606.5 cm^{-1}, which corresponds to the C = C-group; 1388.4-1459.3 cm^{-1}; 1075.3-

1036.2 cm^{-1}, which corresponds to the complex group C – O – C; 894.6-896.4 cm^{-1}, which corresponds to the group $(R)_2$- C = C-H;

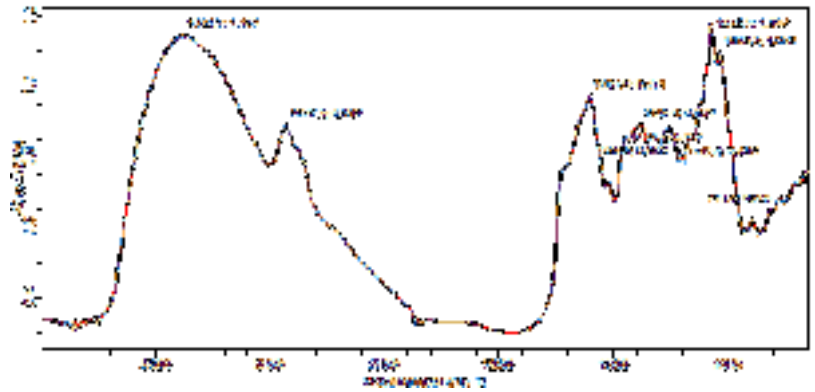


Fig. №33 Stevia 100

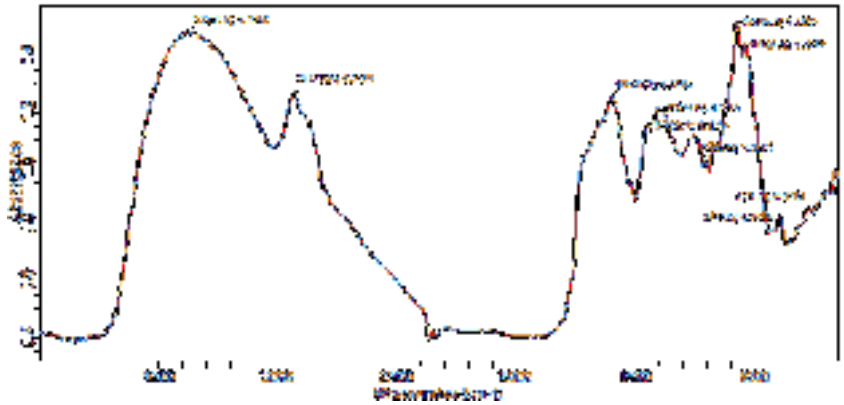


Fig. №34 Stevia 200

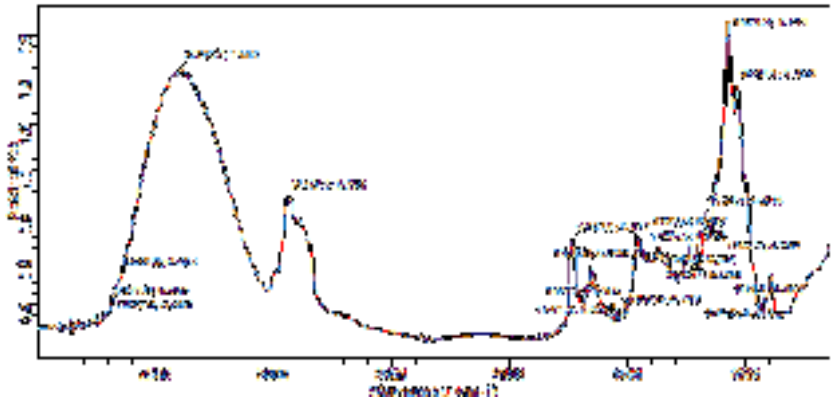


Fig. №35 Stevia 300

During the processing of Stevia preparations, we compared various preparations obtained at different stages of purification from the mixtures and identified them according to the level of sweetness, which is 100, 200 and 300 times sweeter than sugar. The Stevia preparation 300 is a white diterpenoid glycoside without impurities.

The research of the preparation has shown that Stevia 100 and 200 are characterized by almost the same absorption, and the difference is in the level of intensity; Stevia 300 has a change in the background of absorption, what allowed us to conduct a study in the infrared spectrum to determine the purity of the preparation obtained from Stevia. In particular, 2350-3200 cm^{-1}, 1150-1800 cm^{-1}, 950-700 cm^{-1}, the intensity of the absorption spectrum was reduced almost 2 times. Stevia 300 also contained waves with an absorption background: 1075.3-1036.2 cm^{-1}, what corresponds to the complex ester group C-O-C, the complex ester group 1735.1 cm^{-1} C = O and 3377.0-3388 , 2 cm^{-1}, which corresponds to the OH group, clearly indicating a higher content of diterpenoid glycosides in this preparation.

CHAPTER 10. Production of tablets from the products obtained from Stevia.

In order to give a consumer view to preparations of various purities, obtained during the processing of Stevia leaves, we have developed some technologies for the production of tablets (including effervescent tablets).

LFA Tablet Press of TDP-6s company Desktop Tablet Press with tablet equipment)

Equipment and materials necessary for tabletting were purchased with funds allocated by the grant “Low-calorie sweet tablets” SIG / 23/1/2015 sponsored by the “Education, Science and Technological Development Foundation for Tomorrow's Success”. There have been obtained tablets weighing 0.1 g, the sweetness of which is equivalent to 1 teaspoon of sugar.

CONCLUSIONS

1. There have been studied the chemical composition of 4 different varieties (species) of Stevia leaves, introduced in Georgia. A preparation has been obtained from Stevia leaves and 12 diterpene glycosides have been identified using HPLC-UV, RI and UPLC-PDA, MS methods: aglycone - [M-H +] - m / z 319, [M-H -] - m / z 317 steviol; steviol-glucoside - [M-H -] - m / z 479; steviol di-glucoside - [M-H -] - m / z 625; steviol biozid - [M-H -] - m / z 641; treviol triglucoside [M-16] [M-H -] - m / z 787; stevioside - [M-H -] - m / z 803; tetra-glucoside steviol, i.e. rebaudioside A - [M-H -] - m / z 965; mono-rhamnoside-triglucoside steviol, i.e. rebaudioside D - [M-H -] - m / z 1127; C - [M-H -] - m / z 949; steviol tetra-glucoside, i.e. rebaudioside D - [M-H -] - m / z 965; steviol tetra-glucoside, i.e. rebaudioside F - [M-H -] - m / z 935; tri-glucoside steviol, i.e. Dulcid A - [M-H -] - m / z 787; 8 phenolic compounds: mono-caidoyl quina chlorogen acid - [M-H -] - m / z 353; mono-caidoyl quina acid - [M-H -] - m / z 353; 3,5-di-capoyl-quina acid - [M-H -] - m / z 515; 4,5-di-capoyl-quina acid - [M-H -] - m / z 515; quercetin-galactoside - [M-H -] - m / z 463; rutin - [M-H -] - m / z 609; Quercetin-rhamnoside - [M-H -] - m / z 447; Quercetin-Pentoside - m / z 433; Quercetin-galactoside - m / z 463;
2. The oil composition of Stevia leaf has been studied and the dominance of C18 carboxylic acid has been determined; it accounted for more than 50% of total fat content. The following acids have been identified from the oil: C 18- Linolenic acid (C18:2n6c), Cis-Linolic Acid (ω-6), gamma-Linolenic acid (C18:3n6) γ-cis-Linolenic acid (ω -6), α-Linolenic acid C18:3n3) α –Linolenic acid (ω -3)
3. The quantitative content of steviol-glycosides of Stevia leaves of different varieties, as well as the preparations, obtained from them, have been studied. It has been established that a dominant compound of sweet glycosides of Stevia rebaudiana leaves is stevioside, which is up to 6-7% in leaves. Adult leaves accumulate more sweet compounds.
4. The essential oils have been obtained from Stevia leaves; among the found 65 components there have been identified 19 and 6 of them were defined as dominant ones: Thymol methyl (3.891%), Silphinene (11.685%), a-Longipinene (30.730%), Modheph-2-ene(5.757%), a-Isocomene (2.366%), E-Caryophyllen (1.694%), a-Thujene, a-Pinene, y-Terpinene, Nonanal, 1,8-eucalyptol, Perilaldehyde, Undecanal, 2.6-Dodecadien, a-Humulene, a-Sellnene, (E,E)-a-Farnes, b-Cadinene, Z-Caryophyllen.
5. The antioxidant activity of Stevia leaves and the products, obtained from them, was determined by the DPPH method. It has been established that the antioxidant activity of Stevia leaves is almost the same for different varieties, however, it varies significantly during processing. The preparation, which is 100 times sweeter than sucrose, is the most active (only 0.015 mg exhibits DPPH inhibition); the antioxidant activity of the preparation, which is 200 times sweeter, is reduced by almost 7 times (0.107 mg), and in a completely purified preparation (300 times sweeter) antioxidant activity is 45 times less than at the beginning.

6. There has been developed a method of supercritical pressure fluid extraction (SFE) treatment of Stevia leaves. It became possible to fractionate the leaves of biologically active compounds of different composition.
7. A study, using the conductometric detector of cationic chromatography of Stevia leaves, has shown that the total amount of basic cations of Stevia leaves is about 5%, and in the preparation, which is 100 times sweeter than sugar, their amount is slightly more than 5%. The subsequent refining of the preparation at the first stage causes a sharp increase in the number of cations; their number is 8% higher in the preparation, which is 200 times sweeter than sugar; and in the preparation of Stevia, which is 300 times sweeter than sugar, the total amount of cations is up to 0.3%. The dominant cation is potassium, the content of which is 80% more than all cations.
8. Studies of infrared spectroscopy of various preparations, obtained from Stevia leaves, have shown that Stevia 100 and 200 are actually similar in composition, while Stevia 300 has an excellent absorption spectrum, which indicates that it is the most purified of impurities. In particular, 2350-3200 cm^{-1}, 1150-1800 cm^{-1}, 950-700 cm^{-1}, the intensity of the absorption spectrum decreases by almost 2 times. In addition, in Stevia 300 the absorption intensity increases on waves: 1075.3-1036.2 cm^{-1}, which corresponds to the group of complex esters of C-O-C, the group of complex esters of 1735.1 cm^{-1} C = O and 3377.0-3388.2 cm^{-1}, which corresponds to the OH group, this characteristic is directly proportional to the content of diterpenoid glycosides.
9. Technological modes of tabletting the obtained products have been developed. There has been carried out chemical analysis of preparations.

The works published on the basis of Dissertation:

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3. **R. Davitadze**, A. Kalandia Antioxidant activity of stevia products. IX International Conference ,,Bioantioksidant''. Moscow 2015
4. **R. Davitadze**, A. Kalandia Stevia (Stevia Rebaudiana) antioxidant activity of leaf. International scientific-practical conference. Kutaisi 2014
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6. **R. Davitadze**, M. Vanidze, A. Kalandia Sweet tea. Association of Professional Chemists. Third International Conference of Young Chemists ICYC-2012. Tbilisi. 2013
7. **R.Davitadze**, A.Kalandia, M.Vanidze. Stevia - the future of the sugar. The Technical University of Professional Chemists Association. Second International Conference of Young Chemists ICYC-2012. Tbilisi, 2012

Participation in International Scientific Conferences:

1. **R. Davitadze**, A. Kalandia Antioxidant activity of stevia products. IX International Conference ,,Bioantioksidant''. Moscow 2015
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