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Chemical composition of leaf extracts of *Stevia rebaudiana* Bertoni grown experimentally in Vojvodina

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Abstract: The chemical composition of leaf extracts of *Stevia rebaudiana* Bertoni, grown for the first time on an experimental field near Zrenjanin, was examined by GC–MS. The tested plant material was harvested in September of 2002. To analyze the chemical composition of the lipophilic components of the plant leaves, essential oils and ethyl acetate extract were isolated. Qualitative analysis of the essential oil obtained by hydrodistillation showed that among the identified 88 compounds, the majority were mono- and sesquiterpenes (50 types identified). By analysing the ethyl acetate extract, the presence of fatty acids (present as free and as esters), *n*-alkanes, *n*-alkenes, cyclic alkanes, alcohols, aldehydes, ketones, *etc*. was ascertained. Sesquiterpenes prevailed among the terpenes (50 types identified). Further constituents identified in ethyl acetate extract included sterols. Nerol, β-cyclocitral, safranal, aromadendrene, α-amorphene and T-muurolol were identified for the first time in this species, with match values over 90 %. Taking into consideration that these terpenes were identified for the first time in this species, it is obvious that *Stevia rebaudiana* grown in this area possesses certain specific characteristics that can be ascribed to cultivation on a domestic plantation.

Keywords: *Stevia rebaudiana* Bertoni; essential oil; ethyl acetate extract; GC–MS; composition.

INTRODUCTION

Stevia rebaudiana Bertoni belongs to the family Compositae (Asteraceae)[1,2] and is one of only two of the 154 members of the genus *Stevia* producing sweet steviol glycosides.[1] *Stevia rebaudiana* is a small bush that originates from Paraguay.[2] The intensive sweet taste of its leaves has been well known to local Guarani Indians for centuries. In the first place, *Stevia rebaudiana* is known as *yerba*

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dulce in South America, since native Latin American nations called it by many different names: *caá-êhê*, *azuca-caá, kaá-hê-E* and *ca-a-yupe*.[3,4] Most of these names in different ways point to the sweet taste of the leaves of *Stevia rebaudiana*, a property which has been the subject of numerous botanical, physiological and chemical investigations.

Today, exceeding South American continent, all over the world *Stevia rebaudiana* represents a new crop from which natural high-potency non-caloric sweeteners can be obtained. The two main glycosides are stevioside and rebaudioside A, the latter being even 150–320 times sweeter than sucrose.[1] Since numerous studies have established certain effects of *Stevia rebaudiana* and its extracts on the human organism, it has become interesting for the pharmaceutical industry. Among the therapeutic activities attributed to it, hypotensitive regulation, hypoglycemic, antimicrobial and contraceptive activities can be listed.[4–8] As a result, *Stevia rebaudiana* has become rather widespread over a wide range of climatic locations around the world and can apparently be successfully grown under different cultivation conditions. Since the chemical composition of extracts of the leaves of *Stevia rebaudiana* is dependent on the applied conditions of plant cultivation, they have become the subject of many research projects.

The increasing importance of the essential oils in various domains of human life (pharmacy, cosmetics, as well as food and drinks industries) has made this field very interesting for chemical investigations.[9–13] Considering the current research of *Stevia rebaudiana* essential oil, it may be observed that it has been insufficiently investigated. Namely, to the best of our knowledge, there have only been a few studies concerning the composition of *Stevia rebaudiana* essential oil. Fujita *et al.*[9] examined the essential oil of *Stevia rebaudiana* plants collected in several zones of Japan and found that they mainly contained the sesquiterpenes: β-caryophyllene, *trans*-β-farnesene, α-humulene, δ-cadinene, caryophyllene oxide, nerolidol and an unidentified alcohol and the monoterpenes: linalool, terpinen-4-ol and α-terpineol. Martelli *et al.*[10] identified 54 components of a steam distillate of dried plant leaves from Brazil. The main components were caryophyllene oxide and spathulenol, making up 43 % of the overall content. Cioni *et al*.[11] examined the composition of the essential oil of the aerial parts of five different *Stevia rebaudiana* genotypes from Brazil and Paraguay cultivated in the coastal area of Tuscany (Italy). Forty different components were identified and the main constituents in all the samples were spathulenol, caryophyllene oxide, β-caryophyllene and β-pinene.

In order to determine the lipophilic constituents of *Stevia rebaudiana* leaves provided by the Steviafarma Industry (Maringá, RP, Brazil), Yoda *et al.*[12] used supercritical fluid extraction with carbon dioxide. They identified six classes of compounds, *i.e.*, sesquiterpenes, alcohols, labdanic diterpenes, aliphatic hydrocarbons, sterols and triterpenes. In the investigated extract, the main compounds

were diterpenes, of which the most abundant was austroinulin. The other labdanic diterpene represented in a higher level was jhanol. Significant portions of the contents of non-polar components belonged to the hydrocarbons *n*-tetracosane and *n*-pentacosane.

A survey of the pertinent literature showed that no *Stevia rebaudiana* plant material grown in our region has hitherto been examined, except for a material obtained by an *in vitro* procedure of propagation.[14]

In view of the above, the objective of this work was to study the chemical composition of the essential oil and ethyl acetate extract of leaves of *Stevia rebaudiana* Bertoni from Brazil grown on a domestic plantation.

EXPERIMENTAL

Plant material

Stevia rebaudiana leaves (seeds originating from Brazil) were harvested from a domestic plantation near Zrenjanin, Vojvodina. The experimental cultivation of *Stevia rebaudiana* in Vojvodina commenced in 2001, by propagating from seeds. In accordance with the local climate conditions, the seedlings were grown in a glasshouse prior to the growth season. The seedlings were transplanted to the field in the middle of May and harvested in September of 2002.

Extraction

The essential oil of *Stevia rebaudiana* leaves was obtained by hydrodistillation. An amount of 100 g of dried (at 105 °C to constant mass) ground leaves was heated with 1.0 l of water. The distillation was run for 150 min and the essential oil collected in 2.0 ml of *n*-hexane. Subsequently, 1.0 ml of fresh *n*-hexane was added and after separation, the *n*-hexane layer was dried with anhydrous sodium sulphate; the dried filtrate was refrigerated until examination.

The ethyl acetate extract was prepared by heating 500 g of dried and ground plant leaves with 5.0 l of ethyl acetate at the boiling temperature. The treated material and the solvent were left over night and then, after the filtration and vaporization to dryness, the extraction process was repeated with a further 5.0 l of the same solvent. The dry residues after the first (31.30 g) and second (5.56 g) extraction were mixed to make a pooled ethyl acetate extract.

The analytical scheme for the isolation and analysis of the lipophilic constituents of *Stevia rebaudiana* leaves is presented in Fig. 1.

Column chromatography

Dry ethyl acetate extract (30 g) was after the appropriate preparation applied to a silica gel column (Merck, 0.05–0.2 mm). A gradient elution was performed by increasing the concentration of ethyl acetate in benzene from 0 to 25 % (Table I). A constant flow of 1.5 l/24 h was used. The elution was performed continually and 1.5 l volume fractions were collected daily. Each fraction was evaporated to dryness under vacuum.

Thin layer chromatography

Thin layer chromatography (TLC) was used to monitor the qualitative composition of the fractions of the ethyl acetate extract obtained by column chromatography. TLC was performed on 20 cm×20 cm glass plates precoated in our laboratory with silica gel. Silica gel (20 g) was suspended in distilled water (50 ml) and the suspension was applied onto the glass plates using Desaga equipment. The coated plates were dried in the air at room temperature for 24 h,

followed by activation of the plates by drying at 100 °C for 1 h. Small portions of each collected fraction were dissolved in a small volume of ethyl acetate and then applied onto silica gel plates and examined by TLC using benzene as the mobile phase. The chromatograms were observed after spraying with vanillin spray and drying at 130 °C for 10 min.

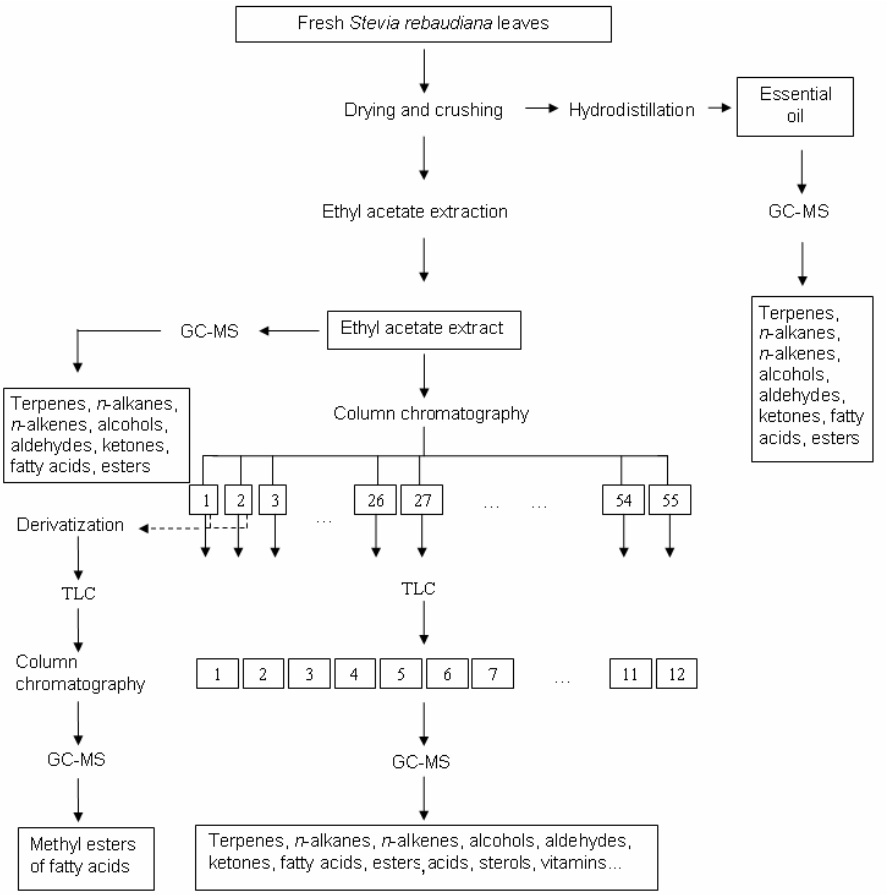


Fig. 1. Analytical scheme for the isolation and analysis of the lipophilic constituents of *Stevia rebaudiana* leaves.

TABLE I. Fractions of ethyl acetate extract eluted by varying polarity of the mobile phase

Composition of mobile phase ethyl acetate–benzene (v/v)	Fraction eluted
0:100	1–28
5:95	29–34
10:90	35–41
15:85	42–47
20:80	48–52
25:75	53–55

Derivatization

The first two ethyl acetate fractions obtained by column chromatography (Fig. 1) were derivatized. To two vessels with 100 mg of elementary sodium dissolved in 100 ml of absolute methanol, 1.00 g of each of the first two ethyl acetate fractions were added under stirring. As the TLC analysis showed their composition to be similar, the two solutions were combined and the resulting solution was filtered and treated with a cationic exchange resin (Purolite C 100E) under constant stirring. After attaining a pH 4–5, the solution was filtered and evaporated to dryness. The mass of the dry residue was 0.45 g. The dry residue was dissolved in 4.0 ml of benzene, of which 3.5 ml were fractionated on a small silica gel column, and the remaining 0.50 ml was kept for TLC observation of fractionation. The elution of methyl esters of fatty acids was realized by passing 200 ml of benzene through silica gel column, followed by the same volume of 25 % ethyl acetate in benzene, for elution of alcohols. Both the collected fractions were evaporated to dryness and the first one was dissolved in benzene, and the second in 25 % ethyl acetate in benzene, the undissolved residues being discarded. After repeating the evaporation to dryness, first fraction, containing methyl esters of fatty acids, was dissolved in 2.0 ml of *n*-hexane, and second containing alcohols, in 2.0 ml of ethyl acetate, to be used for GC–MS analysis.

Gas chromatography–mass spectrometry

The analyses were performed on an Agilent Technologies 6890N gas chromatograph, equipped with a 19091S-433 HP-5MS capillary column (30 m×0.25 mm, 0.25 μm film thickness); the carrier gas was He (43.2 ml min^{-1}). In the analysis of essential oil, the operating conditions were: column temperature 50 °C (1.0 min), 5.0 °C min^{-1} to 100 °C, 9.0 °C min^{-1} to 200 °C (2.89 min), run time 25 min; 5.0 μl of the essential oil solution were injected in the splitless mode at a temperature of 250 °C. The temperature regime in the analysis of ethyl acetate extracts was the following: column temperature 50 °C, 22 °C min^{-1} to 130 °C (1.0 min), 12 °C min^{-1} to 280 °C (12.86 min), run time 30 min, with an injector temperature of 250 °C. A volume of 5.0 μl of ethyl acetate extract solution in *n*-hexane (5.0 mg extract/ml *n*-hexane) was injected onto the column, also in the splitless mode. The Agilent 5973N mass selective detector was equipped with an Agilent 7683 autoinjector; the carrier gas was helium at a constant flow of 1.1 ml min^{-1}. The detector temperature was 230 °C. Ionization was performed by electrons at 70 eV; the scan range was 50–550 *m*/*z*.

Identification procedure

The data was collected with GCD ChemStation (G1074A HP, Version A.00.00) software, which enabled comparison of their mass spectra with those from the Wiley 275.1 MS library. An all-mass range of *m*/*z* 50–550 was used in the matching equation for identification.

RESULTS AND DISCUSSION

Essential oil

The GC–MS total ion current (TIC) chromatograms of the essential oil are shown in Fig. 2. After software processing of the mass fragmentation data and their comparison with the mass spectra from the library, 88 different components were identified in the essential oil of *Stevia rebaudiana* leaves. The identified components are presented in Table II, from which it can be seen that the composition of the essential oil is complex and rich in mono- and sesquiterpenes. Namely, of the 88 identified components, 52 were terpenes. The essential oil was

characterized by a high content of sesquiterpenes, a smaller amount of monoterpenes, and a few diterpenes, namely, 18 monoterpene, 32 sesquiterpene compounds and only two diterpene compounds – alcohol phytol and neophytadiene.

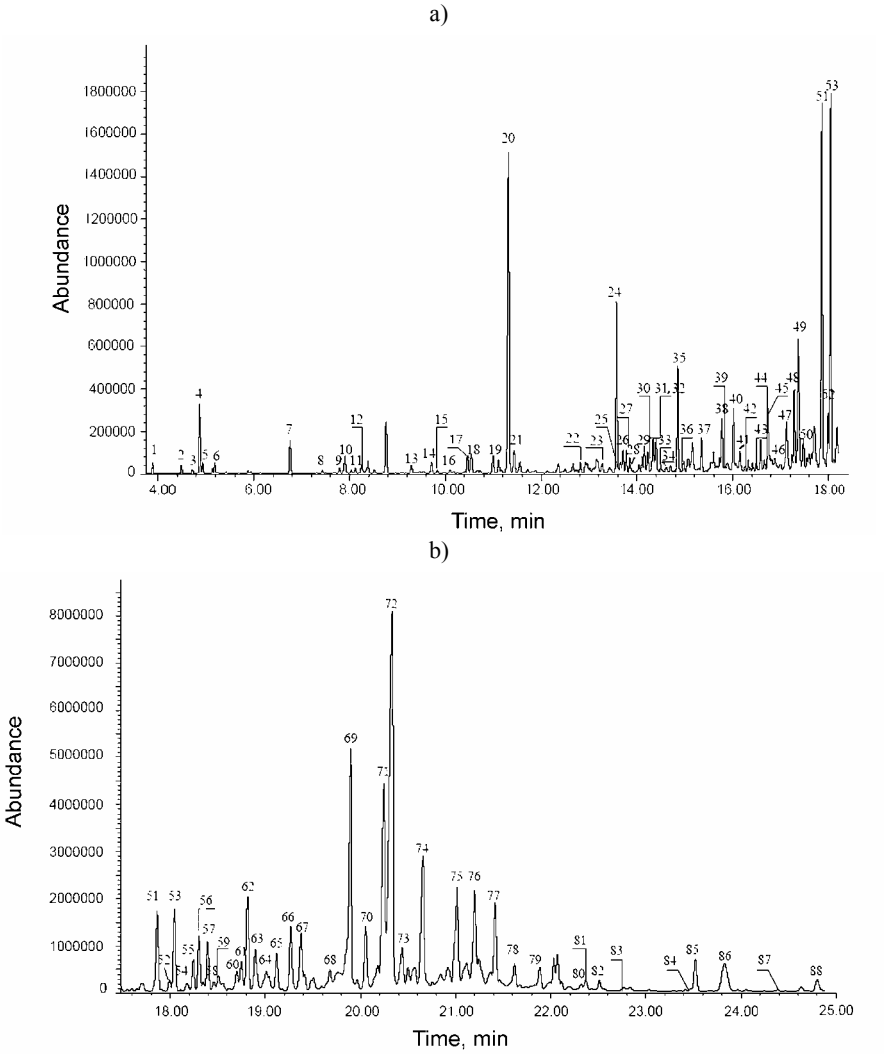


Fig. 2. GC–MS TIC chromatogram of the essential oil of *Stevia rebaudiana* leaves: a) 3.5–18 min; b) 17.5–25 min. Identification of the peaks is given in Table II.

In the group of monoterpenes, three compounds were present that had not previously been reported as constituents of *Stevia rebaudiana*. They are monoterpene nerol and monoterpene aldehydes *β*-cyclocitral and safranal. Safranal, a biologically very active substance, is the main component of the essential oil of saffron (*Crocus sativus* L.).[15,16]

TABLE II. Identification of peaks from Figs. 2a and 2b

Symbol	Compound	Match value %	Symbol	Compound	Match value %
1	*n*-Hexanal	83	45	1,1,6-Trimethyl-1,2--dihydronaphthalene	90
2	Furfural	90	46	Decanoic acid	97
3	5-*tert*-Butyl-1,3--cyclopentadiene	91	47	α-Copaene[s]	99
4	*trans*-2-Hexenal	97	48	*tans*-β-Damascenone[s]	90
5	*cis*-3-Hexenal	96	49	β-Bourbonene[s]	98
6	1-Hexanol	83	50	15-Methyltricyclo[6.5.2(13,14).0(7,15)]-pentadeca-1,3,5,7,9,11,13-heptaene	83
7	α-Pinene[m]	97	51	β-Caryophyllene[s]	99
8	Benzaldehyde	91	52	Germacrene D[s]	92
9	Sabinene[m]	97	53	(–)-*endo*-2,6-Dimethyl-6-(4-methyl-3--pentenyl)bicyclo[3.1.1]hept-2-ene	93
10	1-Octen-3-ol	80	54	Aromadendrene[s]	91
11	6-Methyl-5-hepten--2-one	96	55	Geranylacetone[s]	86
12	2,4-Heptadienal	94	56	β-Farnesene[s]	97
13	Limonene[m]	98	57	α-Humulene[s]	99
14	Benzeneacetaldehyde	90	58	3,4,4a,7,8,8a-Hexahydro-1,1,3,6--tetramethyl-3-vinyl-1*H*-2-benzopyran	80
15	*trans*-β-Ocimene[m]	97	59	*allo*-Aromadendrene[s]	99
16	2-Octenal	83	60	α-Elemene[s]	93
17	3,5-Octadiene-2-one	91	61	α-Curcumene[s]	97
18	*cis*-Linalool oxide[m]	91	62	β-Ionone[s]	96
19	*trans*-Linalool oxide[m]	83	63	β-Selinene[s]	99
20	Linalool[m]	97	64	α-Selinene[s]	98
21	2-Methyl-4-pro-pylimidazol	83	65	β-Bisabolene[s]	98
22	*trans*-2-Nonenal	86	66	α-Amorphene[s]	97
23	Terpinen-4-ol[m]	98	67	δ-Cadinene[s]	98
24	α-Terpineol[m]	91	68	Calacorene[s]	72
25	Methyl salicylate	96	69	Nerolidol[s]	95
26	Myrtenol[m]	94	70	2,6,10-Trimethyl-7,10-epoxy--2,11-dodecadien-6-ol	87
27	Safranal[m]	98	71	Spathulenol[s]	99
28	*n*-Decanal	91	72	Caryophyllene oxide[s]	95
29	1-*p*-Menthen-9-al[m]	94	73	Ledene[s]	89
30	β-Cyclocitral[m]	94	74	Humulene epoxide[s]	83
31	Nerol[m]	94	75	α-Cadinol[s]	91

TABLE II. Continued

Symbol	Compound	Match value %	Symbol	Compound	Match value %
32	*m*-Mentha-1,8-diene[m]	90	76	T-Muurolol[s]	94
33	2-Bornene[m]	93	77	Caryophyllenol II[s]	90
34	2-Hexylbutyrate	78	78, 79	Isomers: 2-(1-cyclohexen-1-yl)-3--hydroxy-5,5-dimethylcyclohex--2-enone	90
35	Geraniol[m]	91		2-(1-cyclohexen-1-yl)-3-hydroxy--5,5-dimethylcyclohex--3-enone	90
36	2,6,6-Trimethyl-cyclo-hexene-1-acetaldehyde	99	80	Tetradecanoic acid (Myristic acid)	98
37	*trans*,*trans*,*trans*-Nona--2,4,6-trienal	87	81	Oplopenone[s]	81
38	Carvacrol[m]	94	82	Caryophylle-3,8(13)-dien-5α-ol[s]	96
39	1,1,6-trimmethyl-1,2,3,4--tetrahydronaphthalene	86	83	Cyclohexadecane	95
40	*trans,trans*-2,4--Decadienal	95	84	Neophytadiene[d]	99
41	*cis*-3-Hexenyl tiglate	90	85	Hexahydrofarnesylacetone[s]	95
42	*n*-Octyl-2-methyl-butyrate	72	86	Phytol[d]	91
43	Methyl anthranilate	83	87	Nonadecane	86
44	*α*-Cubebene[s]	99	88	Farnesylacetone[s]	90

m – monoterpene; s – sesquiterpene; d – diterpene

Sesquiterpenes prevailed in the investigated essential oil as the most numerous group of identified compounds. The most abundant component was caryophyllene oxide, followed by nerolidol and spathulenol. Studies on *Satureja parnassica* oil, which is rich in caryophyllene oxide, has shown antibacterial activity against *Helicobacter pylori*.[17] Furthermore, spathulenol and caryophyllene oxide, identified in *Salvia sclarea* oil, are active against *Staphylococcus aureus*.[18] Nardi[19] showed that the high content of sesquiterpenes and, particularly, the presence of *β*-caryophyllene, spathulenol and caryophyllene oxide could partially account for the antimicrobial activity of aqueous extracts of *Stevia rebaudiana*.

On comparing the present results, obtained by analyzing domestic *Stevia rebaudiana* essential oil, with those of other authors, it can be concluded that there are certain unique properties of the examined oil concerning the composition of sesquiterpenes and monoterpenes.[9–13] One such characteristic is the fact that the sesquiterpenes *α*- and *β*-selinene have been detected in plants from other geographic locations (Japan and Paraguay) but not in the essential oil of leaf. As well as domestically grown *Stevia rebaudiana*, the other *Stevia rebaudiana* lines with

European origins contain sesquiterpene selinene in their leaves. In addition to many sesquiterpenes that have been identified in *Stevia rebaudiana* essential oil, three more were shown as specific constituents of domestic *Stevia rebaudiana*. These compounds are aromadendrene, *α*-amorphene and T-muurolol. Concerning the biological activity of the identified sesquiterpenes, aromadendrene and its isomer ledene (also identified in domestic *Stevia rebaudiana*) have been used as a starting material for the synthesis of fragrances and pheromones.[20] In general, cadinene-type sesquiterpenes have a wide spectrum of biological activity and T-muurolol, which belongs to this group of sesquiterpenes, exhibits significant antifungal activity.[21]

Apart from terpenes in the extracted *Stevia rebaudiana* essential oil (Fig. 2, Table II), numerous organic compounds were identified which contribute to the complex composition of the essential oil, *i.e.*, *n*-alkanes, *n*-alkenes, alcohols, aldehydes, ketones, acids, esters, *etc*.

Ethyl acetate extract

The other investigated extract was the ethyl acetate extract of *Stevia rebaudiana* leaves, which does not include the sweet steviol glycosides.[22–25] Since chloroform and ethyl acetate have been successfully applied as purifying solvents in the extraction of the sweet components from *Stevia rebaudiana* leaves, this procedure enabled the isolation of the other types of components.

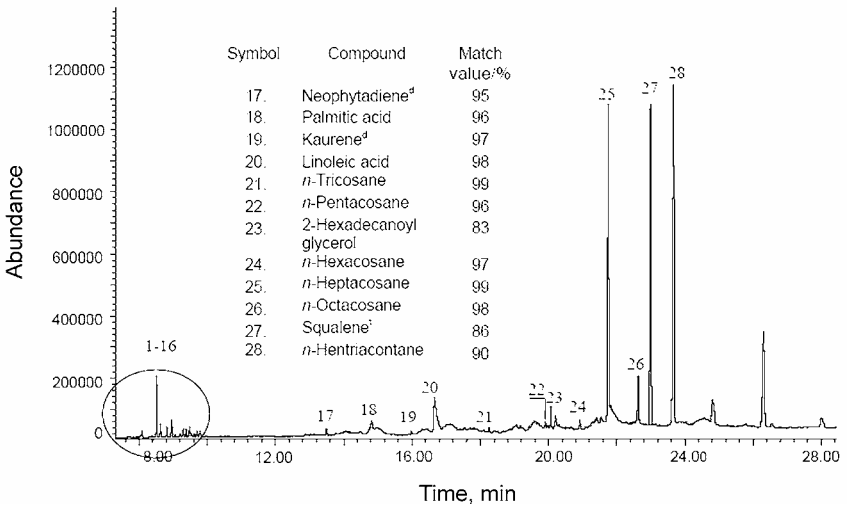
The GC–MS analysis of the ethyl acetate extract of *Stevia rebaudiana* leaves showed the presence of *n*-alkanes, *n*-alkenes, fatty acids and many terpenes (Fig. 3).

In order to characterize the components of the ethyl acetate extract more specifically, it was fractionated by column chromatography. 55 fractions were collected, which were preliminarily examined by thin layer chromatography. Similar TLC chromatogram fractions were pooled into separate groups, so that the final number of samples to be analyzed by GC–MS was 12 (Table III). As can be seen, the first 28 fractions, which were eluted with benzene in the column chromatographic separation (Table I), corresponding to the first 7 samples (Table III), made up about 80 % of the total content, which suggests these compounds were of a non-polar character.

A comparison of the TLC chromatograms of the first two fractions of ethyl acetate extract with TLC chromatograms of refined sunflower oil and *Maclura pomifera* extract showed the presence of fatty acids,[26] as a result of which, it was decided to determine the fatty acids in these fractions. In order to release fatty acid, the first two collected fractions were derivatized after a treatment which led to the alkaline hydrolysis of the fatty acid esters. Separation of the methyl esters of the fatty acids from their alcoholic components was achieved by column chromatography. The fraction containing the methyl esters of the fatty acids was

also examined by GC–MS (Fig. 4). As can be seen, the GC–MS analysis enabled the identification of methyl esters of both saturated and polyunsaturated fatty acids.

a)



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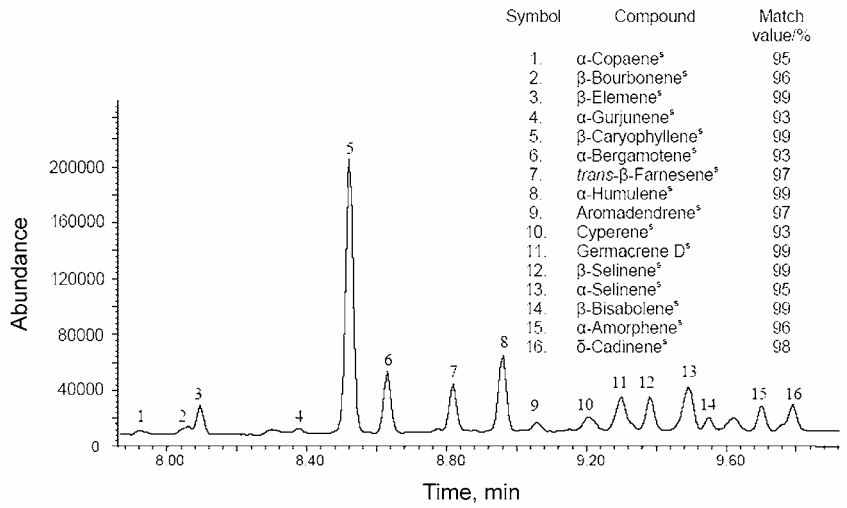

Fig. 3. a) GC–MS TIC chromatogram of the ethyl acetate extract of *Stevia rebaudiana* leaves; b) enlarged encircled segment of A (s – sesquiterpene; d – diterpene; t – triterpene).

Further investigation of the fractions of ethyl acetate extract gave a more complete picture about the rest of lipophilic components present in *Stevia rebaudiana* leaves. Namely, in the GC–MS TIC chromatogram of the ethyl acetate extract recorded before separation by column chromatography, only 28 compounds (Fig. 3) were identified, while after separation 161 compounds were identified.

This is certainly a consequence of the significantly larger concentrations of analyte in the latter case.

TABLE III. Distribution of the fractions collected in 12 samples and their masses

Sample	Fraction collected	Mass, g
1	1	4.81
2	2	6.93
3	3	2.92
4	4–9	3.90
5	10–15	0.96
6	16–21	2.61
7	22–32	3.26
8	33–37	0.52
9	38–39	0.31
10	40–46	1.81
11	47–50	0.81
12	51–55	0.72

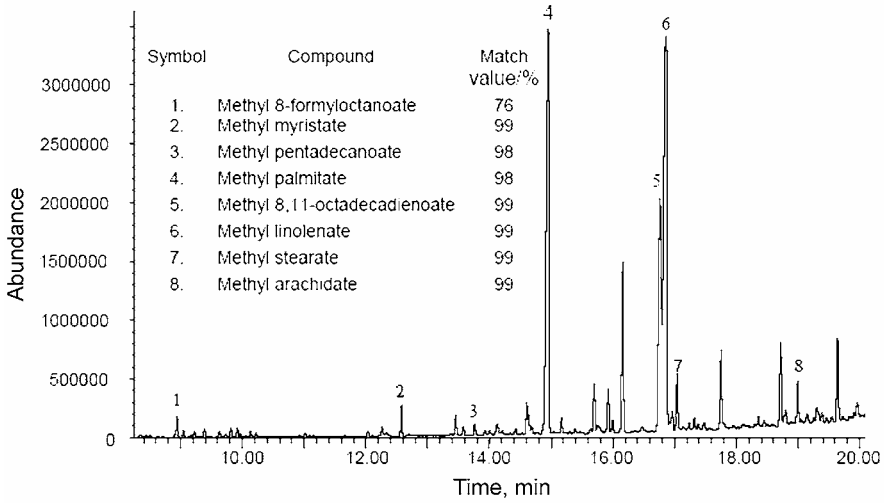

Fig. 4. GC–MS TIC chromatogram of the methyl esters of fatty acids from *Stevia rebaudiana* leaves.

A comparison of the terpene composition of *Stevia rebaudiana* essential oil and its ethyl acetate extract showed the absence of monoterpenes in the ethyl acetate extract, whereas 35 sesquiterpenes were identified. Most of them were already determined in *Stevia rebaudiana* essential oil, whereas the rest were specific for the ethyl acetate extract. Those newly identified in the ethyl acetate extract were cyperene, γ-gurjunene, globulol, ledol, α-isocedren-15-al and oxygen and methyl derivatives of ionone. Sesquiterpenes were the most abundant

group of compounds in the ethyl acetate extract prior to fractionation and in the first two fractions collected during column chromatography. In these fractions, sesquiterpenes were accompanied mostly with fatty acid esters, *n*-alkanes, *n*-alkenes and ketones. It is interesting to note that the sesquiterpenes β-caryophyllene, α-humulene and β-farnesene, which were identified in both the essential oil and the ethyl acetate extract of *Stevia rebaudiana*, were also found in the essential oil of hop. Namely, they are 3 of the 4 most common compounds of hop aroma, their ratios being distinctive for the variety.[27]

Nine diterpenes were identified in the ethyl acetate extract. This group of compounds were also more numerous than the diterpenes identified in the essential oil of *Stevia rebaudiana*. Identification of diterpene kaur-16-ene indicates the presence of kaurene-type diterpenes. This group of diterpenes represents a skeleton for forming steviol glycosides, the main sweet components in *Stevia rebaudiana* leaves. Apart from this, a manoyl oxide-type of diterpenes was also determined. Manoyl oxide, along with *epi*-13-manoyl oxide, has been biologically investigated as a natural product of different plant species, since it shows certain anti-inflammatory activity and also exhibits an important activity against specific parasites.[28] The labdane diterpene sclareol, which was also present, is also a biologically active compound. Some recent investigations were concerned with the cytotoxic and antitumor activity of liposome-incorporated sclareol against cancer cell lines and human colon cancer xenografts.[29] It was found that liposomes incorporate sclareol at a drug to lipid mole ratio of 0.43, suggesting an incorporation efficiency of almost 80 %, and induced a reduced growth rate of human colon cancer tumours (HCT116) developed in SCID mice, without any significant side effects.

In view of the low polarity of first collected fractions, the total content of triterpenes in stevia leaves was eluted by benzene. Squalene, amyrin and lupenon, together with triterpene alcohols, constitute this group of compounds.

With the same non-polar solvent, almost the total content of *n*-alkanes, *n*-alkenes, alcohols, aldehydes and ketones was eluted. Only a few of them were collected straight after the mobile phase was made more polar by the addition of ethyl acetate to benzene. The *n*-alkanes identified in the investigated extract were mostly long-chain *n*-alkanes C_{13}–C_{31}, with only two cycloalkanes, *i.e.*, cyclotetracosane and cyclooctacosane. The two most common *n*-alkanes were *n*-heptacosane and *n*-hentriacontane. Similar results were also obtained in a study of the *n*-alkanes from plants of the genus *Achillea*.[30] Unsaturated hydrocarbons, apart from the mentioned terpenes, are represented by a group of C_{14}–C_{23} *n*-alkenes, whereas alcohols are a dominant group among oxygen derivatives in the ethyl acetate extract.

A white crystalline substance was isolated from the eighth ethyl acetate fraction. GC–MS analysis of the very light white crystal showed that it consisted mainly of a long-chain aliphatic alcohol, *n*-eicosanol.

Among the 14 identified acids, saturated and unsaturated fatty acids, *i.e.*, palmitic, oleic, stearic, linoleic, *etc*., were the most abundant.

The non-polar fractions of the ethyl acetate extract, obtained using column chromatographic separation with benzene as the eluent (Tables I and III), contained most of the identified esters. Prior to the application of column chromatography, one monoglyceride, 2-monopalmitate, was identified in this extract, whereas after fractionation it was possible to identify esters of formic and adipic acid, followed by fatty acid esters and unsaturated monoglycerides (1-monolinolenin and 1-monolinolein). These fractions were also richer in aromatic compounds, mostly alkyl derivatives of naphthalene, and vitamin E was identified in some of them.

All the sterols and stanols identified in the wax of *Stevia rebaudiana* leaves were also present as constituents of the fractions of the extract eluted with benzene. The compounds identified were sitosterol and a derivative of stigmastanol. This type of phytosterols plays an important role in human biology. By lowering the level of low-density lipoprotein cholesterol, these phytosterols protect the cardiovascular system of humans.[31]

The polarity of the eluting solvent was gradually changed by adding ethyl acetate to benzene. A smaller number of compounds were identified in the more polar fractions of ethyl acetate extract due to the nature of the rather complex mixture. During column chromatography, oxygen derivatives of sesquiterpenes and diterpenes were eluted with a polar mixture of solvents. Only a few *n*-alkanes, *n*-alkenes, alcohols and ketones, together with several acids, were identified in the more polar fractions of the investigated extract.

A group of lactones was specific for the more polar solvent fractions. Loliolide and dihydroactinidiolide represent degradation products of carotenoides.[32,33] Moreover, the identified imide of maleic acid, 2-ethyl-3-methylmaleimide, might be present as a by-product of the photo-oxidation of chlorophyll,[34] since this pigment is evidentially present in *Stevia rebaudiana* leaves.

CONCLUSIONS

A qualitative analysis of the essential oil from leaves of domestic *Stevia rebaudiana* Bertoni showed that mono- and sesquiterpenes prevailed (50 types identified) among the 88 identified compounds. By analyzing the ethyl acetate extract, the presence of terpenes, fatty acids (present as free and esters), *n*-alkanes, *n*-alkenes, cyclic alkanes, alcohols, aldehydes, ketones, *etc*., was ascertained. Among the terpenes (50 types identified), sesquiterpenes prevailed. Further constituents identified in the ethyl acetate extract include sterols. Taking into account that some of the compounds were identified for the first time in this species (nerol, β-cyclocitral, safranal, aromadendrene, α-amorphene and T-muurolol), it is obvious that *Stevia rebaudiana* grown in this area possesses certain specific

characteristics, which can be ascribed to the cultivation conditions on the domestic plantation, *i.e.*, location, climate, soil and plant growing conditions.

This examination is a contribution to a better understanding of all the aspects and potential uses of *Stevia rebaudiana* leaves and their extracts. It becomes even more important in view of the development and improvement of plant cultivation in Serbia. The results also support the necessity of research and use of *Stevia rebaudiana* extract as the source of natural products different from steviol glycosides, since it is rich in terpenes, alcohols and fatty acids.

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ИЗВОД

ХЕМИЈСКИ САСТАВ ЕКСТРАКАТА ЛИСТА *Stevia rebaudiana* BERTONI ЕКСПЕРИМЕНТАЛНО ГАЈЕНЕ У ВОЈВОДИНИ

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Применом GC–MS методе, испитан је хемијски састав екстраката листа *Stevia rebaudiana* Bertoni, по први пут експериментално гајене на плантажи у околини Зрењанина. Испитивани биљни материјал је пожњевен септембра 2002. године. За анализу хемијског састава липофилних компоненти листова стевије изоловано је етарско уље и етилацетатни екстракт. Квалитативном анализом етарског уља, добијеног хидродестилацијом, утврђено је да је богато моно- и сесквитерпенима, тј. да од 88 идентификованих компоненти 50 су моно- и сесквитерпени. Анализом хемијског састава етилацетатног екстракта домаће *Stevia rebaudiana* утврђено је да се она одликује присуством масних киселина (присутних у виду естара и слободних киселина), *n*-алкана, *n*-алкена, цикличних алкана, алкохола, алдехида, кетона и др. Од терпена (идентификовано 50 врста) доминирају сесквитерпени. Поред тога у етилацетатном екстракту су регистровани и стероли. Терпени нерол, β-циклоцитрал, сафранал, аромадендрен, α-аморфен и Т-муролол су први пут идентификовани у овој биљној врсти са слагањем добијених масених спектара са оним из Wiley библиотеке преко 90 %. Имајући у виду да су наведени терпени по први пут идентификовани у овој биљној врсти, очигледно је да *Stevia rebaudiana* гајена на нашим просторима поседује извесне специфичности које се могу приписати условима гајења на домаћој плантажи.

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REFERENCES

1. J. E. Brandle, A. N. Starratt, M. Gijzen, *Can. J. Plant Sci.* **78** (1998) 527, http://sci.agr.ca/ /london/faq/stevia_rev_e.htm (April 29, 2007)
2. D. J. Midmore, A. H. Rank, *A New Rural Industry – Stevia – To Replace Imported Chemical Sweeteners*, A report for the Rural Industries Research and Development Corpo-

ration, Australia, RIRDC Project No UCQ-16A, 2002, pp. 1-3, http://www.rirdc.gov.au/ /reports/NPP/02-022.pdf (June 2, 2007)
3. W. H. Lewis, *Econ. Bot*. **46** (1992) 336
4. D. Mowrey, *Life with Stevia: How Sweet it is!*, Nutritional and Medicinal Uses, Health Freedom Resources, 1992, http://www.formerfatguy.com/articles/stevia.asp (April 29, 2007)
5. P. Chan, B. Tomlinson, Y. J. Chen, J. C. Liu, M. H. Hsieh, J. T. Cheng, *Br. J. Clin. Pharmacol.* **50** (2000) 215
6. P. B. Jeppensen, S. Gregersen, S. E. D. Rolfsen, M. Jepsen, M. Colombo, A. Agger, J. Xiao, M. Kruhøffer, T. Ørntoft, K. Hermansen, *Metabolism* **52** (2003) 372
7. G. Planas Mazzei, J. Kuc, *Science* **162** (1968) 1007
8. F. Dozono, N. Sato, U. S. Patent, 5262161 (1993)
9. S. Fujita, K. Taka, Y. Fujita, *Yakugaku Zasshi* **97** (1977) 692 [C.A. **87**/1977 122621v]
10. A. Martelli, C. Frattini, F. Chialva, *Flavour Frag. J.* **1** (1985) 3
11. P. L. Cioni, I. Morelli, L. Andolfi, M. Macchia, L. Ceccarini, *J. Essent. Oil Res*. **18** (2006) 76
12. S. K. Yoda, M. O. M. Marques, A. J. Petenate, M. A. A. Meireles, *J. Food Eng.* **57** (2003) 125
13. http://www.rain-tree.com/stevia-chemicals.pdf (April 29, 2007)
14. M. Popović, N. Mimica-Dukić, S. Mezei, O. Gašić, M. Gavrilović, N. Mrkovački*, Arh. Farm.* **50** (2000) 304
15. H. Hosseinzadeh, H. R. Sadeghnia, *J. Pharm. Pharmaceut. Sci*. **8** (2005) 394
16. A. N. Assimpoulou, Z. Sinakos, V. P. Papageorgiou, *Phytother. Res*. **19** (2005) 997
17. O. Tzakou, H. Skaltsa, *Planta Med.* **69** (2003) 282
18. A. Ulubelen, G. Topcu, C. Eris, U. Soenmez, M. Kartal, S. Kurucu, C. Bozok-Johansson, *Phytochemistry* **36** (1994) 971
19. U. Nardi, *Erboristeria Domani* **6** (1996) 58
20. Y. M. A. W. Lamers, *Ph.D. Thesis*, Wageningen University, Wageningen, 2003
21. C. L. Wu, S. C. Chien, S. Y. Wang, Y. H. Kuo, S. T. Chang, *Holzforschung* **59 (**2005) 620
22. Y. Oshima, J. Saito, H. Hikino, *Tetrahedron* **42** (1986) 6443
23. Y. Oshima, J. Saito, H. Hikino, *Phytochemistry* **27** (1988) 624
24. A. Rajbhandari, M. F. Roberts, *J. Nat. Prod.* **46** (1983) 194
25. N. F. Komissarenko, A. I. Derkach, I. P. Kovalyov, N. P. Bublik, G. A. Chermeneva, A. G. Kotov, V. V. Zinchenko, *Rast. Resursy* **30** (1994) 53
26. S. Filip, *M.Sc. thesis*, University of Belgrade, Faculty of Chemistry, Beograd, 2001 (in Serbian)
27. Z. Zeković, I. Pfaf-Šovljanski, O. Grujić, *J. Serb. Chem. Soc.* **72** (2007) 81
28. A. García-Granados, E. Liñán, A. Martínez, F. Rivas, C. M. Mesa-Valle, J. J. Castilla--Calvente, A. Osuna, *J. Nat. Prod.* **60** (1997) 13
29. S. Hatziantoniou, K. Dimas, A. Georgopoulos, N. Sotiriadou, C. Demetzos, *Pharmacol. Res.* **53** (2006) 80
30. R. Palić, T. I. Eglinton, B. C. Benitez-Nelson, G. Eglinton, J. Veličković, G. Stojanović, *J. Serb. Chem. Soc.* **64** (1999) 443
31. C. A Vanstone, M. Raeini-Sarjaz, W. E. Parsons, P. J. H. Jones, *Am. J. Clin. Nutr.* **76** (2002) 1272
32. T. Y. P. Bonnie, Y. M. Choo, *J. Oil Palm Res.* **2** (1999) 62
33. J. S. Sinninghe Damsté, M. P. Koopmans, *Pure Appl. Chem*. **69** (1997) 2067
34. Q. Xian, H. Chen, H. Liu, H. Zou, D. Yin, *Environ. Sci. Pollut. Res.* **13** (2006) 233.