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Gas chromatography–mass spectrometry analysis of free and glycoconjugated aroma compounds of seasonally collected *Satureja montana* L.

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Abstract

The present work examines the content and composition of glycoconjugated and free volatile aroma compounds in the plant material of savory, depending on the stage of plant development. Free volatile compounds (essential oil) as well as volatile aglycones obtained after the enzymatic hydrolysis of isolated glycosides were analyzed by coupled gas chromatography–mass spectrometry (GC–MS). Thirty-six compounds were identified in the essential oil and 17 compounds among the volatile aglycones. The main components of essential oil (yield 0.80–1.46% w/w, SD = 0.02–0.03) were thymol (30.88–46.02%, SD = 0.40–0.45), *p*-cymene (7.10–13.48%, SD = 0.19–0.27), γ-terpinene (7.57–9.74%, SD = 0.19–0.23) and carvacrol (3.81–6.86%, SD = 0.05–0.07). The main aglycones (yield 58.4–95.6 mg kg^{-1}, SD = 1.5–3.0) were: thymoquinone (27.55–32.93%, SD = 0.38–0.40), eugenol (8.35–19.44%, SD = 0.06–0.23), cis-3-hexene-1-ol (2.38–9.73%, SD = 0.04–0.22) and thymol (4.07–7.81%, SD = 0.05–0.06). Other aglycones in lower percentages were *p*-cymen-8-ol, α-terpineol, geraniol, benzyl alcohol, 2-methoxy-4-ethyl-6-methylphenol, 1-octen-3-ol, methyl salicylate, 3,5,5-trimethyl-4-(3-hydroxy-1-buthenyl)-2-cyclohexene-1-one, carvacrol and other compounds. The results show moderate similarity in the chemical composition of oil and volatile aglycones. In the aglycones fraction, the contents of thymoquinone and thymol decreased with maturation of the plant, and contents of eugenol, nerol, geraniol, increased in the same time. This is mainly in agreement with seasonal variations of the main oil components.

Keywords: *Satureja montana* L.; Essential oil; Glycosides of volatile compounds; Seasonal variations; Enzymatic hydrolysis; GC–MS

1. Introduction

Satureja montana L. subsp. *montana* (*Lamiaceae*), winter savory, is a well-known aromatic and medicinal herb. It is a deciduous perennial shrub (20–30 cm high). This plant grows wild on rocky, barren and sunny places (in high amount) along the Adriatic coast and the submediterranean part of Croatia. Savory is one of the best honey plants. The honey of savory is famous in folk remedies for bronchitis. This plant contains various biologically active constituents such as essential oil, triterpenes (Escudero, Lopez, Rabanal, & Valverde, 1985), flavonoids (Thomas-Barberan, Huisain, & Gil, 1987) and rosmarinic acid (Reschke, 1983). The plant, essential oil and extracts are used as folk remedies for many diseases (with bactericidal, carminative, digestive, expectorant, fungicidal, laxative, antidiuretic, sedative and antioxidant activity). The essential oil of this plant is applied in flavouring condiments, relishes, soups, sausages, canned meats, and in spicy table sauces. In recent study, Pepeljnjak, Stanić, and Potočki, (1999) examined the antimicrobial activity of ethanolic extract of savory on 11 species of bacteria and five species of fungi. The plant and plants extract showed high antioxidant effect (Madsen, Nielson, Bertelsen & Skibsted, 1996). The extract and essential oil of savory was investigated on diuretic activity in rats (Stanić & Samardžija, 1993). Ciani et al. (2000) examined antimicrobial properties of savory essential oil on pathogenic and spoilage yeasts. According to Lawrence (1979), the value of savory oil is in its high carvacrol content and its fresh, spicy phenolic notes reminiscent of oregano and thyme. All these plant species contain

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essential oils with phenol compounds (thymol and carvacrol) as their major components. The content of thymol and carvacrol in savory is variable and depends on the origin and vegetative stage of the plant (Kuštrak, Kuftinec, Blažević, & Maffei, 1996; Slavkovska, Jančić, Bojović, Milosavljević, & Djoković, 2001; Stanić, Petričić, & Blažević, 1991). The tea and extracts of this plant among many groups of natural compounds can contain free and glycoconjugated aroma compounds. Glycosidically bound volatiles were identified in many aromatic and nonaromatic plants (Crouzet & Chassagne, 1999). Alcohols, phenols and mercaptans often exist in plants as their glycosides. While the presence of carbohydrate moieties in such compounds is responsible for their specific characteristics and transport in the body fluids, the glycones do not have any significant effect on the biological activity of glycosides. These glycosides may be precursors for the biological production of flavours. They are generally water soluble, nonvolatile and flavourless. They can however, be hydrolysed or pyrolysed into a glycon part and an aglycone with flavouring properties. The study of glycoconjugated and free aroma compounds might be of some pharmacological interest as well as for food additives as it may add extra flavour value to the end product. The aim of this study was to determine the composition of volatile aglycones released from water soluble glycosides and to show their possible similarity with the free aroma compounds of this undoubtedly valuable medicinal and spice plant.

2. Experimental

2.1. Reagents

All of the solvents employed (p.a. grade) and β-glucosidase were purchased from Fluka Chemie, Buchs, Switzerland. Octyl-β-D-glucopyranoside, silica gel for column chromatography (Kieselgel 60, 0.040–0.063 mm), precoated silica plates (Kieselgel 60, thickness 0.2 mm) for thin layer chromatography, ammonia, calcium carbonate and sodium sulphate were obtained from Merck, Darmstadt, Germany.

2.2. Plant material

Savory was collected in the submediterranean region of south Croatia (near Sinj) during the summer 2001 from wild-growing populations. The collection of plant material was prior to flowering (in June and August) and during flowering (in September). After flowering (in October), the plant material was not collected, because in this stage of development, the plant contained very small leaves. Air-drying of the plant was performed in shady place for twenty days at room temperature. For this investigation leaves and flowers were used. The voucher specimens are deposited at Department of Organic Chemistry, Faculty of Chemical Technology, Split, Croatia.

2.3. Isolation of essential oil and glycosides

One hundred grams of plant material was submitted, for 3 h, to a simultaneous extraction of water soluble compounds and hydrodistillation of the essential oil in Clevenger type apparatus. Octyl-β-D-glucoside (500 μg; internal standard for glycosides) and calcium carbonate powder (10 g; for neutralisation of eventually existing plant acid) were added to water intended for simultaneous extraction and hydrodistillation. After distillation the essential oil was separated, dried over Na_2SO_4 and stored. The aqueous extract was decanted, and the residual plant material was extracted once more with boiling water. The pooled aqueous extracts were concentrated to 30 ml in a rotating evaporator under reduced pressure at 50 °C. Ballast components (saccharides, proteines and other compounds) were removed by precipitation with 100 ml ethanol. The remaining ethanol–water solution (filtrate) was then concentrated to 20 ml in the same way. To the obtained syrup, 30 ml of ethanol and a few drops of concentrated ammonia were added for precipitation of acidic ballast compounds. Finally, the purification was performed by flash chromatography on a silica gel column applying ethyl acetate:ethanol: ammonia 6:3:1 v/v/v. It is possible that small part of the glycosides was not extracted and was loss during the extract purification. TLC analyses showed the absence of free carbohydrates in the glycosidic fraction. The obtained glycosidic fraction was concentrated to dryness, dissolved in a citrate buffer (pH 5.5; 5 ml) and the remaining free terpenes and hydrophobic compounds were removed with pentane-dichloromethane extraction as described in a previous paper (Mastelić & Kuštrak, 1997).

2.4. Hydrolysis of glycosides

In a typical experiment, β-glucosidase from almonds (20 mg, 5–8 U/mg) was added to the glycosidic solutions along with 3 ml pentane for trapping the liberated aglycones. The hydrolysis was carried out for 70 h at 30 °C with the mixture being shaken occasionally. After hydrolysis, the pentane layer was separated and remaining aglycones were extracted from the aqueous layer with pentane (10×2 ml). The combined pentane extracts were dried (Na_2SO_4, 0.3 g) and after separation of pentane layer, the solid Na_2SO_4 was washed twice with 5 ml ether. Combined pentane and ether extracts were concentrated to final volume of 0.5 ml (by careful fractional distillation), and 1 μl was used for GC–MS analysis.

2.5. Gas chromatography–mass spectrometry analysis

Analysis was performed on a GC–MS Hewlett-Packard (model 5890 with a mass selective detector model 5971A, Hewlett Packard, Vienna, Austria) using two columns with different polarity of stationary phases. GC operating conditions: column HP-20M (Carbowax 20M, Hewlett Packard, Vienna, Austria), 50 m×0.2 mm i.d., film thickness 0.2 μm; column temperature programmed from 70 °C isothermal for 4 min, to 180 °C at a rate of 4 °C min^{-1}; column HP-101 (Dimethylpolysiloxane, Hewlett Packard, Vienna, Austria), 25 m×0.2 mm i.d., film thickness 0.2 μm; column temperature programmed from 70 °C isothermal for 2 minutes, to 200 °C at a rate of 3 °C min^{-1}; carrier gas: helium; flow rate: 1 ml min^{-1}; injector temperature: 250 °C; volume injected: 1 μl; split ratio: 1:50. MS conditions: ionisation voltage: 70 eV; ion source temperature: 280 °C; mass range: 30–300 mass units.

2.6. Identification and quantitative determination of components

Individual peaks were identified by comparing their retention indices to those of authentic samples, as well as by comparing their mass spectra with those stored in data base (Wiley library) as well as with spectra published by Adams (1995). Determination of the percentage composition was based on peak area normalisation without use of correction factors. The content of aglycones was calculated from GC peak areas related to the GC-peak area of 1-octanol (liberated from octyl-β-glucoside). Preliminary GC–MS analysis shoved the absence of 1-octanol as potential algycone of savory. The oil yield was determined by gravimetry. The component percentages in Tables 1 and 2 were calculated as the mean-value of the component percentages on column HP-20M and HP-101 for duplicate analysis.

3. Results and discussion

3.1. Essential oil

Essential oil of savory was subjected to a detailed GC–MS analysis for the purpose of determination of its chemical composition variability depending on the plant development stage. Table 1. shows the seasonal variations of the yields and compositions of savory essential oil. The yield of oil was highest in June (1.46%, SD = 0.03) at beginning of summer. With growing of the plant yields decreased in August and September (0.80%, SD = 0.02). Thirty-six compounds were identified representing 96.6–99.8% of the total oil. The major oil components were: thymol (30.88–46.02%, SD = 0.40–0.45), *p*-cymene (7.10–13.48%, SD = 0.19–0.27), γ-terpinene (7.57–9.74%, SD = 0.19–0.23), carvacrol (3.81–6.86%, SD = 0.05–0.07), carvacrol methyl ether (4.01–5.11%, SD = 0.05–0.05) and thymol methyl ether (3.83–5.11%, SD = 0.05–0.07). The essential oil also contained smaller percentages of borneol (2.36–4.05%, SD = 0.04–0.05), β-caryophyllene (1.66–3.75%, SD = 0.03–0.04), α-terpinene (1.71–3.87%, SD = 0.04–0.05) and myrcene (1.42–2.60%, SD = 0.04–0.05).

Phenols as thymol and carvacrol and their methyl ethers are main components of the oil (42.53–60.25%). Other oxygen-containing oil compounds with smaller amounts are monoterpne alcohols as geraniol, borneol, linalool, nerol and esters as geranyl and thymyl acetate. Furthermore, the main components of the oil monoterpene hydrocarbons are *p*-cymene, γ-terpinene, α-terpinene, myrcene and the main representatives of oil sesquiterpene hydrocarbons are β-caryophyllene, ledene, aromadendrene, β-bisabolene and δ-cadinene (Table 1).

The highest content of thymol was identified in young plant (June), and highest carvacrol content during flowering in September. The content of *p*-cymene increased with the maturation of the plant, and contents of γ-terpinene, α-terpinene decreased with maturation. The contents of alcohols (linalool, geraniol, borneol, nerol), aldehydes (geranial, neral) and ester (geranyl acetate) increased with the maturation of the plant. We do not identified geraniol, geranial, nerol, neral and geranyl acetate in the young plant material in distinction of other collected materials. The contents of thymol methyl ether and carvacrol methyl ether are approximately constant. Thymol and carvacrol represent isomeric monoterpene phenols, which are biosynthesised *via p*-cymene and γ-terpinene. Similar results of essential oil analysis for this plant, but from different locality (also in Croatia) were reported early (Miloš, Radonić, Bezić & Dunkić, 2001). They identified carvacrol as main component of the oil. It is well known that environmental conditions, stage of development and especially chemotype of plant can affect composition and content of essential oil.

3.2. Glycoconjugated aroma compounds

The contents of volatile aglycones in dried plant material were 58.4–95.6 $mgkg^{-1}$ (SD = 1.5–3.0). The highest content (95.6 $mgkg^{-1}$) had the plant in flowering, and the smallest plant in August (58.4 $mgkg^{-1}$). Young plant had medium-value (73.2 mg kg^{-1}, SD = 2.5). Seventeen aglycones were identified. The results are shown in Table 2. Among the aglycones were identified aliphatic alcohols, terpene compounds, derivates of phenylpropanes, C_{13}-norisoprenoids and nitrogen heterocyclic compound 1-H-indol. The main aglycones were: thymoquinone (27.55–32.93%, SD = 0.38–0.40), eugenol (8.35–19.44%, SD = 0.06–0.23), cis-3-hexene-1-ol

Table 1
Yield and percentage composition of savory essential oil depending on the stage of plant development

No.	Component	Prior to flowering				In flower		Mode of identification
		June 20		August 30		September 15		
		%	SD	%	SD	%	SD	
1	α-Thujene	1.77	0.04	1.92	0.04	1.58	0.03	I_1, I_2, MS
2	α-Pinene	0.52	0.02	1.29	0.03	0.99	0.03	I_1, I_2, MS
3	β-Pinene	0.10	0.01	t	/	t	/	I_1, I_2, MS
4	δ-3-Carene	0.40	0.02	0.10	0.01	t	/	I_1, I_2, MS
5	Myrcene	2.20	0.04	2.60	0.05	1.42	0.04	I_1, I_2, MS
6	α-Terpinene	3.87	0.05	2.34	0.05	1.71	0.04	I_1, –, MS
7	β-Phellandrene	0.44	0.02	0.35	0.02	t	/	I_1, I_2, MS
8	Limonene	1.68	0.04	t	/	t	/	–, I_2, MS
9	γ-Terpinene	9.49	0.22	9.74	0.23	7.57	0.19	I_1, I_2, MS
10	*p*-Cymene	7.10	0.19	13.48	0.27	12.36	0.25	I_1, I_2, MS
11	Terpinolene	t	–	0.29	0.02	t	/	–, I_2, MS
12	Alloocimene	0.96	0.03	–	/	–	/	I_1, –, MS
13	1-Octen-3-ol	0.55	0.02	0.87	0.03	0.63	0.02	I_1, –, MS
14	Sabinene hydrate	–	/	0.36	0.02	–	/	–, I_2, MS
15	Terpinen-4-ol	0.14	0.01	0.11	0.01	t	/	I_1, –, MS
16	Linalool	0.37	0.02	3.05	0.04	3.15	0.04	I_1, I_2, MS
17	Thymol methyl ether	5.11	0.07	3.83	0.05	4.99	0.06	I_1, I_2, MS
18	Carvacrol methyl ether	4.60	0.05	4.01	0.05	5.11	0.05	I_1, I_2, MS
19	β-Caryophyllene	3.75	0.04	1.66	0.03	2.64	0.04	I_1, I_2, MS
20.	Aromadendrene	0.38	0.02	0.20	0.01	0.22	0.01	I_1, I_2, MS
21	Neral	–	/	t	/	0.45	0.02	I_1, –, MS
22	Humulene	0.23	0.02	0.10	0.01	–	/	–, I_2, MS
23	Borneol	2.36	0.04	4.05	0.05	3.62	0.04	I_1, I_2, MS
24	Ledene (Viridiflorene)[a]	1.28	0.03	0.29	0.02	0.34	0.02	–, I_2, MS
25	β-Cubebene	0.30	0.02	t	/	t	/	I_1, I_2, MS
26	Geranial	–	/	0.55	0.02	0.28	0.02	I_1, –, MS
27	β-Bisabolene	0.82	0.02	0.45	0.02	0.55	0.02	I_1, I_2, MS
28	Geranyl acetate	–	/	3.03	0.03	3.38	0.03	I_1, I_2, MS
29	δ-Cadinene	0.44	0.02	0.12	0.01	0.23	0.01	I_1, I_2, MS
30	Nerol	–	/	0.54	0.02	0.49	0.02	I_1, –, MS
31	Geraniol	–	/	6.43	0.05	4.15	0.04	I_1, I_2, MS
32	Thymol acetate	0.17	0.01	–	/	–	/	–, –, MS
33	p-Cymen-8-ol	–	/	0.11	0.01	–	/	I_1, –, MS
34	Sphatulenol	0.34	0.02	–	/	–	/	–, –, MS
35	Thymol	46.02	0.45	30.88	0.42	35.41	0.40	I_1, I_2, MS
36	Carvacrol	4.52	0.06	3.81	0.05	6.86	0.07	I_1, I_2, MS
	Identified (%)	99.81		96.56		98.13		
	Oil yield (%)	1.46		0.80		0.80		
	SD	0.03		0.02		0.02		

I_1, retention indices on HP-20M; I_2, retention indices on HP-101; SD, standard deviation; /, not calculated SD; MS, mass spectra; –, not detected; t, traces <0.1%. The compounds are ordered according to retention indices on HP-20M.

[a] Tentatively identified with mass spectrum MW = 204. *m*/*z*: 107(100), 105 (92), 93(80), 161(65), 119(58), 135(50), 79(50), 204(32), 189(32), 175(22), 147(20).

(2.38–9.73%, SD = 0.04–0.22), thymol (4.07–7.81%, SD = 0.05– 0.06) and *p*-cymene-8-ol (5.74–7.85%, SD = 0.05–0.06). 3,5,5-Trimethyl-4-(3-hydroxy-1-buthenyl)-2-cyclohexene-1-ol, 2-methoxy-4-ethyl-6-methylphenol, 2,4,4-trimethyl-3-(3-hydroxybuthyl)-2-cyclohexene-1-one, α-terpineol, 2-phenylethanol, benzyl acohol, geraniol, 1-octen-3-ol, carvacrol and indol were identified in smaller percentages. Aliphatic C_{6-8}-alcohols, geraniol, benzyl acohol, eugenol, 2-phenylethanol and α-terpineol were identified in many other aromatic plants. Aromatic plants with thymol and carvacrol in essential oil often contain thymol and carvacrol glycosides among their water soluble compounds. Thymoquinone was identified as aglycone in *Origanum vulgare* and other plants (Jerković, Mastelić, & Miloš, 2000; Miloš & Radonić, 1996), probably as thymohydroquinone glycoside. After the enzymatic hydrolysis liberated thymohydroquinone oxidises spontaneously to thymoquinone. A similar

Table 2
Content and composition of savory volatile aglycones depending on the stage plant development

No.	Component	Prior to flowering				In flower		Mode of identification
		June 20		August 30		September 15		
		%	SD	%	SD	%	SD	
1	*cis*-3-Hexene-1-ol	9.73	0.22	2.38	0.04	4.27	0.05	I_1, I_2, MS
2	1-Octen-3-ol	1.45	0.03	1.00	0.02	t	/	I_1, I_2, MS
3	α-Terpineol	2.98	0.04	3.20	0.09	2.49	0.03	I_1, I_2, MS
4	Thymoquinone	32.93	0.40	27.55	0.38	27.98	0.38	I_1, I_2, MS
5	Methyl salicylate	0.69	0.02	0.67	0.02	0.82	0.02	I_1, –, MS
6	Nerol	–	/	0.90	0.02	1.07	0.02	I_1, –, MS
7	Geraniol	–	/	4.40	0.05	4.59	0.05	I_1, I_2, MS
8	Benzyl alcohol	4.37	0.04	3.09	0.04	4.70	0.05	I_1, I_2, MS
9	2-Phenylethanol	1.23	0.03	2.13	0.03	4.03	0.04	I_1, I_2, MS
10	*p*-Cymen-8-ol	5.74	0.05	7.85	0.06	5.85	0.05	I_1, I_2, MS
11	Eugenol	8.35	0.06	19.44	0.23	17.40	0.22	I_1, I_2, MS
12	Thymol	7.81	0.06	4.18	0.05	4.07	0.05	I_1, I_2, MS
13	Carvacrol	0.87	0.02	0.73	0.02	t	/	I_1, I_2, MS
14	Indol	1.25	0.03	1.58	0.03	3.33	0.05	I_1, I_2, MS
15	3,5,5-Trimethyl-4-(3--hydroxy-1-buthenyl)--2-cyclohexene-1-ol	2.49	0.03	4.86	0.04	5.67	0.04	–, I_2, MS
16.	2-Methoxy-4-ethyl--6-methylphenol	5.44	0.04	6.58	0.05	4.28	0.04	–, I_2, MS
17.	2,4,4-Trimethyl-3-(3--hydroxybuthyl)-2--cyclohexene-1-one	–	/	2.16	0.04	–	/	–, I_2, MS
	Identified (%)	85.33		92.70		90.55		
	Aglycone yield (mg kg^{-1})	73.2		58.4		95.6		
	SD	2.5		1.5		3.0		

I_1, retention indices on HP-20M; I_2, retention indices on HP-101; SD, standard deviation; /, not calculated SD; MS, mass spectra; –, not detected; t, traces $<0.1\%$. The compounds are ordered according to retention indices on HP-20M.

reaction was noted in hydrojuglone-β-glucoside hydrolysis (glycoside of hydroquinone) to juglone (quinone) by glucosidase and nonenzymatically oxidation (Duroux, Delmotte, Lancelin, Keravis, & Jay-Allemand, 1998). Aglycones as cis-3-hexene-1-ol and 1-octen-3-ol are aliphatic alcohols. They can be originated of fatty acid catabolism. Eugenol, 2-methoxy-4-ethyl-6-methylphenol, benzyl alcohol, 2-phenylethanol and methyl salycilate are belonging in phenylpropane derivatives and related compounds. Aglycones with more complex structures as 3,5,5-trimethyl-4-(3-hydroxy-1-buthenyl)-2-cyclohexene-1-ol and 2,4,4-trimethyl-3-(3-hydroxybuthyl)-2-cylohexene-1-one are C_{13}-norisoprenoids, identified early in leaves of *Nicotiana* species (Kodama, Fujimori, & Kato, 1984). They are formed by cleavage of carotenoids. The aglycone 2-methoxy-4-ethyl-6-methylphenol belongs to phenylpropane derivatives and related compounds. Similar compounds (as 2-methoxy-4-methylphenol, 2-methoxy-4-vinylphenol and methoxy vinylphenol isomers) were identified among the aglycones of other plants (Stahl-Biskup, Intert, Holthuijzen, & Schulz, 1993). Among the volatile aglycones indol was also identified in concentration 1.25–3.33%, SD = 0.03–0.03. It is heterocyclic compound with active NH-group and makes N-glycosides, in distinction of other aglycones. Indol as aglycone was identified in *Origanum vulgare* (Mastelić, Miloš, & Jerković, 2000), and in aglycones of tea leaves (Wang, Kubota, Kobayashi, & Juan, 2001). The remain aglycones as α-terpineol, nerol, geraniol, *p*-cymen-8-ol, thymol and carvacrol are monoterpene compounds.

By comparison of the chemical composition of the oil (Table 1) and aglycones (Table 2) six compounds were established to be identical: thymol, *p*-cymene-8-ol, geraniol, 1-octen-3-ol, carvacrol and nerol. These results show moderate correlation between the chemical composition of oil and volatile aglycones. Furthermore, the contents of thymoquinone and thymol decreased with maturation of the plant, and contents of eugenol, nerol and geraniol increcreased in the same time (Table 2). This is mainly in agreement with the seasonal variations of the main essential oil components (Table 1). The conclusions on seasonal effect are not absolute, but indicate the major trends of seasonal changes. Absolute

quantitative study of these compounds as well as their seasonal changes will demand the collection of different batches of plant.

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