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Short communication

Chemical composition and antioxidant effect of glycosidically bound volatile compounds from oregano (*Origanum vulgare* L. ssp. *hirtum*)

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Abstract

The present work examines the content and chemical composition of the glycosidically bound volatiles from oregano as well as their antioxidative properties. The glycosidically bound volatiles amounted to 20 mg kg^{-1} in dried leaves and flowers of oregano. Fourteen volatile aglycones were identified with thymoquinone as the major component. Other important aglycones were benzyl alcohol, eugenol, 2-phenyl-ethanol, thymol, 3-hexen-1-ol and carvacrol. It was found that all of the aglycones have an antioxidant effect when tested by measuring peroxide values of lard stored at 60°C. These results were compared to the antioxidative activity of oregano essential oil, pure thymol, thymoquinone and also to α-tocoferol which is well known among natural antioxidant compounds.

Keywords: *Origanum vulgare* L.; Glycosides; Natural antioxidants; Antioxidant activity

1. Introduction

The application of synthetic antioxidants as inhibitors of lipid oxidation is well known in the food industry. Widely used artificial antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Chan, 1987), are very effective in their role. However, their use in food products has been falling off due to their instability, as well as due to a suspected action as promoters of carcinogenesis (Namiki, 1990; Pokorny, 1991). For this reason, there is a growing interest in the studies of natural additives as potential antioxidants. The antioxidant properties of many herbs and spices are reported to be effective in retarding the process of lipid peroxidation in oils and fatty foods and have gained the interest of many research groups. A number of studies on the antioxidant activities of various aromatic plants have been reported over the last 20 years (Brraco, Loliger & Viret, 1981; Herrmann, Schutte & Muller, 1981; Kramer, 1985; Lagouri, Blekas, Tsimidou, Kokkini & Boskou, 1993).

Shahidi, Janitha and Wanasundara (1992) reported that the antioxidant effect of aromatic plants is due to the presence of hydroxy groups in their phenolic compounds. Tsimidou and Boskou (1994) concluded that among the herbs and spices extensively studied, the plants obtained from the *Lamiaceae* (*Labiatae*) family possess a significant antioxidant activity. Lagouri et al. (1993) studied the antioxidant activity of essential oils and they found that oregano essential oil, rich in thymol and carvacrol, has a considerable antioxidant effect on the process of lard oxidation. In recent studies, Yanishlieva and Marinova (1995) examined the antioxidant activity of hexane extracts of oregano grown in Bulgaria, as well as the mechanism of action of pure thymol and carvacrol (Yanishlieva, Marinova, Gordon & Raneva, 1999). In our previous work (Milos, Mastelic, Jerkovic & Katalinic, in press) the oregano essential oil was fractionated according to acidity and polarity, in order to correlate properly the antioxidant activity to the chemical composition. The fraction containing only thymol and carvacrol exhibited significant antioxidant effect.

Regarding the nonvolatile components, the extracts of oregano have the most effective antioxidant activity among aromatic herbs (Vekiari, Oreopoulou, Tzia & Thomopoulous, 1993). Different groups of researchers (Chevolleau, Mallet, Ucciani, Gamisans & Gruber, 1992; Herrmann et al., 1981; Kramer, 1985) studied oregano alcohol extracts. The antioxidant effect of the

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mentioned extracts is generally due to the presence of rosmarinic and caffeic acid. The study of Banias, Oreopoulou and Thomopoulos (1992) showed that oregano extracts have strong antioxidant effects in stabilizing lard.

In spite of extensive investigations on the antioxidant activity of volatile and nonvolatile compounds of aromatic herbs, very little is known to what extent the glycosides or the glycosidically bound volatile compounds may contribute to the total antioxidant activity. The presence of such compounds in different plants has been well established previously and many publications have dealt with their chemistry and distribution in the plant kingdom (Francis & Allcock, 1969; Grzunov, Mastelic & Ruzic, 1985; Merkx & Baerheim Svendsen, 1990; Stahl-Biskup, Intert, Holthuijezen, Stengele & Schultz, 1993). The glycosides are able to release volatile compounds by the enzymatic or an acid hydrolysis so they can be considered as potential precursors of antioxidant substances in plant materials.

The studies on the antioxidant activity of glycosidically bound volatile compounds of oregano have not been reported to date. The aim of this work was to isolate and identify these compounds as well as to determine their antioxidative properties with respect to lipid oxidation in the lard.

2. Experimental

2.1. Materials

Oregano (*Origanum vulgare* L., ssp. *hirtum*) was collected in central Dalmatia in October 1998. The plant material consisted of flowered tops and stalks (15–20 cm). Air-drying of oregano was performed in a shady place at room temperature for 10 days. The plant material was used for the isolation of the glycosides, immediately after drying. The voucher specimen is deposited in the Laboratory of Organic Chemistry, Faculty of Chemical Technology, Split, Croatia.

The oregano essential oil was isolated by hydrodistillation of the plant material for 3 h (Milos et al., in press). The total of 16 compounds were identified representing 97.6% of the oil. The main components were thymol (40.4%), carvacrol (24.8%) and *p*-cymen (16.8%). The essential oil also contained smaller quantities of γ-terpinene (1.7.%), 1-octen-3-ol (2.1%), borneol (1.2%) and terpinen-4-ol (2.1%). Similar results were reported for oregano of Greek origin (Vokou, Kokkini & Bessiere, 1993). The lard was prepared by heating a fat (pork meat) at 100°C. The isolated lard was filtered and stored at −18°C until further use.

All of the solvents employed were of proanalysis purity and purchased from Fluka Chemie, Buchs, Switzerland. The compounds α-tocoferol, thymol, benzyl alcohol, 2-phenyl ethanol, β-glucosidase and meso-tetraphenylporphyrin Fe (III) chloride complex were obtained also from Fluca Chemie, Buchs, Switzerland. Octyl-D-glucopiranoside, potassium monopersulfate triple salt and anhydrous sodium sulfate were obtained from Merck, Darmstadt, Germany.

2.2. Preparation of thymoquinone

Thymoquinone was prepared by Fe (III) meso-tetraphenylporphyrin catalyzed oxidation of thymol by $KHSO_5$. In a typical experiment (Martins, Neves, Silvestre, Silva & Cavaleiro, 1999), thymol (1 mmol), $KHSO_5$ (0.5 mmol) and the catalyst (10^{-2} mmol) were dissolved in acetonitrile (3 ml). The reaction was performed in a thermostated reaction tube equipped with a magnetic stirring bar at 40°C. Aliquots (10 μl) were withdrawn during the reaction course, poured into pentane (1 ml) and 2 μl were used for the GC–MS analysis. The reaction was stopped when the product yields remained constant after two successive analyses. Thereupon, the reaction mixture was poured into water (3 ml) and extracted with pentane (10 ml). The remained traces of thymol from the pentane layer were washed out by NaOH solution (20%). The pentane layer was dried over an anhydrous sodium sulphate and concentrated in the rotary evaporator (Elektromaterijal, Ljubljana, Slovenia). Thymoquinone has been unambiguously identified as the only one oxidation product by the gas chromatography and mass spectrometry.

2.3. Isolation of glycosides

The glycosides were isolated from 100 g of dried plant material by exhaustive extraction with boiling water for 3 h. The obtained water extract was filtered and the residual plant material was extracted, once more, with 200 ml of boiling water. Pooled extracts were concentrated to dryness in the rotating evaporator, under reduced pressure. The residue was dissolved in ethanol and purified by selective precipitation of the ballast compounds with water and ammonia (Mastelic & Kustrak, 1997). Final purification was performed by flash chromatography on silica gel (30–60 μm, Mallinckrodt Baker B.V., Deventer, The Netherlands) column (length 20 cm; i.d. 2 cm) applying as the eluens the mixtures of ethyl acetate, ethanol and ammonia in 6:3:1 volume ratio. The purified glycosidic fraction was concentrated to dryness and dissolved in 2 ml distilled water. The remaining free terpenes and hydrophobic compounds were extracted 10 times with 5 ml of pentane. Prior to the enzymatic hydrolysis, the absence of any volatile compounds in the glycosidic fraction was tested by thin layer chromatography (TLC) applying the mixtures of ethyl acetate and hexane (in 85:15 volume ratio) as the mobile phase. The layer thickness of the silicagel plates was 0.2 mm (Merck, Darmstadt, Germany).

2.4. Hydrolysis of glycosides and separation of aglycones

β-Glucosidase (20 mg) was added to the glycosidic solutions along with 3 ml pentane for trapping the liberated aglycones. The hydrolysis was carried out for 72 h at 30°C. Occasionally, the mixture was shaken thoroughly by hand. After the hydrolysis, the pentane layer containing the aglycones was decanted off by a vacuum pipette. The remaining aglycones from the aqueous layer were extracted 10 times with 5 ml pentane. The TLC (described in the previous paragraph) and GC–MS analysis of the last pentane extract showed the absence of aglycones and confirmed the completeness of the extraction. The combined pentane extracts were dried over an anhydrous sodium sulfate, then concentrated by reduced pressure to the final volume of 0.5 ml and 1 μl was used for the gas chromatography-mass spectral (GC–MS) analysis.

The aglycone concentrations were calculated, in a parallel experiment, from the GC peak areas related to the GC peak area of 1-octanol (liberated from the internal standard octyl-β-D-glucopyranoside added just before the enzymatic hydrolysis). Preliminary GC–MS analysis showed the absence of 1-octanol as a potential aglycone in the plant material.

2.5. Gas chromatography–mass spectrometry

The analyses of the volatile compounds were run on a Hewlett Packard GC–MS system (GC 5890 series II; MSD 5971A, Hewlett Packard, Vienna, Austria). The fused-silica HP-20 M polyethylene glycol column (50 m × 0.2 mm, 0.2 μm thickness, Hewlett Packard, Vienna, Austria) was directly coupled to the mass spectrometer. The carrier gas was helium (1 ml/min). The program used was 4 min isothermal at 70°C, then 4°C/min to 180°C and 10 min isothermal. The injection port temperature was 250°C and the detector temperature was 280°C. Ionization of the sample components was performed in the EI mode (70 eV).

2.6. Identification and quantitative determination of components

Linear retention indices for all compounds were determined by coinjection of the sample with the solution containing the homologous series of C_8–C_{22} *n*-alkanes (Van Den Dool & Kratz, 1963). Individual constituents (Table 1) were identified by comparing their retention indices: with the library data (Adams, 1995), with the retention indices of the compounds originating from the known plant sources, with those retention indices obtained from the authentic components. The mass spectra of the compounds were compared with Wiley mass spectral database (Hewlett Packard, Vienna, Austria).

2.7. Antioxidant assay

The main sample of lard was transferred to a series of open transparent glass bottles (surface area 7 cm^2). Each bottle contained 10 g of the lard. The isolated aglycones and essential oil as well as pure thymol, thymoquinone and α-tocoferol were separately dissolved in pentane and added to the lard samples, which were melted at 60°C. The samples of lard contained 2000 ppm of each additive. Two replicates for all samples were prepared. All samples were stored at 60°C in the incubator (Instrumentaria, Zagreb, Croatia) out of light. For comparison, a control test without additives was prepared also from the same main sample of lard. Peroxide values (PV) were determined periodically according to the American Oil Chemists Society ([AOCS] 1994) method Ce 8-53.

3. Results and discussion

3.1. Glycosidically bound volatiles

The content of glycosidically bound volatile compounds in dried plant material was 20 mg kg^{-1}. Fourteen aglycones were identified as shown in Table 1. Aliphatic alcohols, terpene compounds and derivatives of phenylpropanes were all present among the volatile aglycones. The major aglycone was thymoquinone

Table 1
Percentage composition of the glycosidically bound volatile compounds of *Origanum vulgare* L

No.	Component	RI[a] (HP-20 M)	Peak area (%)	Mode of identification[b]
1	Unknown	1086	1.5	GC
2	3-Hexen-1-ol	1350	3.4	GC,MS
3	1-Octen-3-ol	1415	1.3	GC,MS
4	Benzaldehyde	1475	0.9	GC,MS
5	Thymoquinone[c]	1690	40.2	GC,MS,CO
6	Methyl salycilate	1714	0.6	GC,MS
7	Unknown	1750	1.8	GC
8	Benzyl alcohol	1812	8.9	GC,MS,CO
9	2-Phenyl ethanol	1851	5.6	GC,MS,CO
10	Eugenol	2094	7.5	GC,MS
11	Thymol	2123	3.5	GC,MS,CO
12	Carvacrol	2149	2.4	GC,MS
13	1-H-Indole	—	0.8	MS
14	2-(p-Metoxyphenyl) ethanol	—	1.3	MS
	Total		79.7	

[a] RI, retention indices relative to C_8–C_{22} *n*-alkanes on the polar HP-20M column.

[b] GC, compared with retention indices of known components; MS, mass spectra; CO, identity confirmed by comparison with authentic compounds.

[c] Mass spectrum of thymoquinone (2-*isopropyl*-5-methylbenzoquinone) MW 164; *m*/*z*: 164(100), 149(49), 136(46), 121(64), 108(18), 93(56), 77(29), 53(30).

(40.2%). Other important aglycones were benzyl alcohol (8.9%), eugenol (7.5%), 2-phenyl-ethanol (5.6%). thymol (3.5%), 3-hexen-1-ol (3.4%) and carvacrol (2.4%). A lot of aglycones (such as linalool, geraniol, nerol, α-terpineol and terpinen-4-ol) considered more or less as ubiquitous in aglycone fractions from the family Lamiaceae (Stahl-Biskup et al., 1993), were not identified among the oregano's aglycone compounds.

The total content of the essential oil (yield = 2.9%) is 1450 times higher than that of the aglycones (yield = 0.002%). The comparison of the chemical composition of the volatiles present as glycosidically bound aglycones to the chemical composition of free volatiles (Milos et al., in press) showed only 3 common compounds (thymol, carvacrol and 1-octen-3-ol). This suggests moderate correlation between the free volatile compounds in the essential oil and those volatiles in the form of glycosidically bound aglycones.

3.2. Antioxidant activity

The aim of this work was to evaluate the antioxidant effectiveness of the volatile aglycones from oregano and to compare it to the antioxidant activity of oregano essential oil as well as to pure thymol, thymoquinone and α-tocoferol. Both aglycones and essential oil as well as pure thymol, thymoquinone and α-tocoferol showed antioxidant effect when tested by measuring peroxide values of lard stored at 60°C. Fig. 1 gives the profiles of peroxide values in the lard assays within three months. In the absence of an antioxidant (control test) the rate of hydroperoxide formation increased sharply after 10 days. The aglycones and essential oil showed similar antioxidant activity and inhibited hydroperoxide formation even after 80 days, while pure thymol and thymoquinone showed lower antioxidant activity. These results were compared to the antioxidant effectiveness of α-tocoferol, a well known natural antioxidant. It is obvious that the aglycones and essential oil inhibited the formation of hydroperoxides more than α-tocoferol. On the other hand, pure thymol as the major component of the essential oil and thymoquinone as the major component among the aglycones, inhibited the formation of hydroperoxides less than α-tocoferol. It appears that the synergy among minor compounds plays an important role. This observation is another confirmation that the use of synergistic mixtures of antioxidants allows a reduction in the concentration of each substrate. It also increases the antioxidant effectiveness when compared with the activity of each separate compound (Abdala & Roozen, 1999).

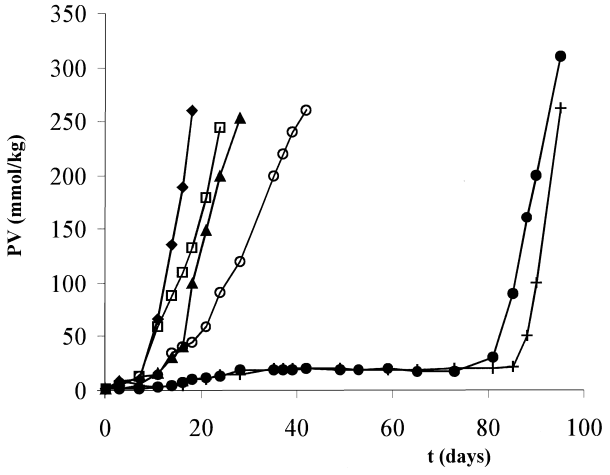

Fig. 1. Effect of *Origanum vulgare* L. on the autoxidation rate of the lard stored at 60°C in the presence of the essential oil (+), volatile aglycones (●), α-tocoferol (○), thymol (▲) and thymoquinone (□); control test without antioxidants (◆). PV- peroxide values.

In the essential oil the antioxidant effect is related to the presence of thymol and carvacrol (Milos et al. in press; Yanishlieva et al., 1999). Among the volatile aglycones, besides thymol (3.5%) and carvacrol (2.4%) which are present in relatively small percentage, thymoquinone (40.2%) as the main component could contribute to the antioxidant activity (Guenther & Althausen 1963) even if quinones are generally themselves mild oxiditing agents. Houghton, Zarka, Delasheras and Hoult (1995) showed the role of thymoquinone as an inhibitor of membrane lipid peroxidation. Recently, two publications have dealt with antitumor and hepatoprotective activity of thymoquinone (Daba & Abdelrahman, 1998; Worthen, Ghosheh & Crooks, 1998).

Thymoquinone and the other glycosidically bound volatiles in the spice plant oregano were found to be potent antioxidants, comparable in activity with its essential oil as well as to widely used natural antioxidant α-tocopherol. This should merit further investigation of the use of oregano glycosidic extracts as natural antioxidants in food which could be interesting for some medicinal aspects also.

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