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OXIDATIVE STABILISATION OF SUNFLOWER OIL BY ANTIOXIDANT FRACTIONS FROM SELECTED LAMIACEAE HERBS

This study reports the effect of antioxidant fractions from rosemary (Rosmarinus officinalis), sage (Salvia officinalis), thyme (Thymus vulgaris) and hyssop (Hyssop officinalis) on the oxidative stability of sunflower oil at elevated temperature. In order to isolate antioxidant fractions, the method of fractional supercritical extraction with carbon dioxide at 35 MPa and 100 °C was applied. Antioxidant fractions were added to sunflower oil at concentrations of 200 mg/kg oil and the samples were stored in an oven maintained at 98 °C. The antioxidant activity of the extracts was determined by measuring peroxide values (PV). Among investigated extracts, the rosemary extract was most effective on retarding lipid oxidation of sunflower oil. The antioxidant activity of the extracts was compared to the activity of butylated hydroxyanisole (BHA) and a commercial rosemary extract Flavor' Plus. On the basis of PV assay, the antioxidant activity of the investigated plant extracts after 12 h of storage at 98 °C followed the order: rosemary extract > BHA > sage extract > Flavor' Plus > thyme extract > hyssop extract.

Key words: peroxide value; rosemary; sage; thyme; hyssop; supercritical extraction.

Lipid oxidation is one of the major forms of spoilage in foods, because it leads to the formation of off-flavours and potentially toxic compounds. It affects the quality of the product due to the loss of a desirable colour, odour, and flavour and reduces the shelf-life. This process also produces reactive oxygen species (ROS) which have been implicated in carcinogenesis, inflammation, early aging and cardiovascular diseases [1]. To prevent lipid oxidation, food products should be kept away from oxygen, stored at low temperatures to retard oxidation reactions or supplied with antioxidants [2]. Common antioxidants in the food, cosmetics and pharmaceutical industries such as butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) are synthetic. The use of synthetic antioxidants is restricted in several countries because of their undesirable long-term toxicological effects, including carcinogenicity [3-5]. Also, the utilization of synthetic antioxidants is limited because consumers are increasingly demanding of additive-free or natural products [6]. The majority of natural antioxidants are phenolic compounds or polyphenols and their antioxidant activity is based on their structure, hydrogen-donating potential and ability to chelate metal ions. They may show higher efficiency than endogenous or synthetic antioxidants [7].

The antioxidant activity of rosemary extracts has been associated with the presence of several phenolic diterpenes such as carnosic acid, carnosol, rosmanol, rosmariquinone and rosmaridiphenol [8-11]. Carnosic acid (Figure 1) is a lipophilic antioxidant that scavenges singlet oxygen, hydroxyl radicals, and lipid peroxyl radicals, thus preventing lipid peroxidation and disruption of biological membranes [12,13]. Its radical scavenging activity is caused by the presence of two *o*-phenolic hydroxyl groups found at C_{11} and C_{12} of the molecule [14]. In a study of 16 compounds isolated from rosemary, Bracco *et al.* [15] concluded that the antioxidant activity of rosemary extracts which were obtained using peanut oil, is primarily related to two phenolic diterpenes, carnosol and carnosic acid. During the storage and extraction of rosemary, carno-

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sic acid is partially converted either into carnosol or into other diterpenes such as rosmanol. Nakatani and Inatani [16] identified rosmanol and carnosol from rosemary and found that both were more effective than α-tocopherol, BHT and BHA using an active oxygen method (AOM). They also reported that rosmanol had greater antioxidant activity than carnosol. Nguyen *et al.* [17] reported that rosemary supercritical extract was more effective than BHA/BHT (1:1) in canola oil and margarine. In the same study [18], the antioxidant activity of rosemary and sage supercritical extracts were higher than the BHA/BHT (1:1) activity in prime steam lard and slightly less in the cases of thyme and oregano. There are more reports on antioxidant activity of rosemary extract obtained by conventional methods (distillation and solvent extraction) in several foods: chicken sausages [18], beef [15,19], pork sausages [20], cured pork fat [21], beef patties [22,23], chicken meat [24] and fish products [25].

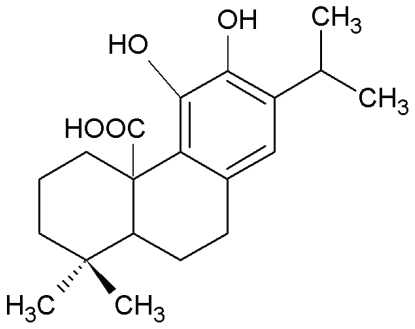

Figure 1. Molecular structure of carnosic acid.

Cuvelier *et al.* [26] separated the major antioxidants in sage by high performance liquid chromategraphy (HPLC). Six major antioxidant compounds were carnosol, carnosic acid, rosmadial, rosmanol, epirosmanol, and methyl carnosate.

The biphenyls and *p*-cumene-2,3-diol in thyme possess significant antioxidant activity [27,28]. Budincevic *et al.* [29] tested ethanol extracts of thyme using tallow and lard as substrates at 60 °C in the Rancimat apparatus. The extracts showed antioxidant effects with the substrates processed at 60 °C but not at 100 °C. Simandi *et al.* [30] isolated a thyme extract by the supercritical carbon dioxide extraction at the pressure of 40 MPa and the temperature of 60 °C. Antioxidant activities of thyme extracts were detected by measuring the peroxide value in sunflower oil at 60 °C, and authors confirmed than the effectiveness of the thyme extract added at the level of 6000 mg/kg oil was equal to that of 1000 mg BHA/kg oil.

Among the medicinal and aromatic plants hyssop is a plant that has not been studied very much. Dapkevicius *et al.* [31] reported that the antioxidant activity of deodorized hyssop extracts was very low in comparison with those of thyme, marjoram and sage. Djarmati *et al.* [32] found a high activity related to rosmanol-9-ethyl ether from the alcoholic extract of the hyssop which was much greater than the synthetic antioxidant agent, butylated hydroxytoluene (BHT). Fernandez-Lopez *et al.* [33] also reported that hyssop inhibited lipid oxidation and degradation of heme pigments in cooked pork meat caused by cooking and storage. In another report, hyssop extracts were found to exhibit prooxidative action and increase conjugated diene formation in sunflower oil [34].

The food industry needs to combat strict regulations and comply with measures for safety, reliability, and standardization of natural products to be consumed as nutrients and food additives. This may be achieved by adopting supercritical fluid extraction (SFE) techniques by which the active ingredients in natural form without degradation or contamination could be produced. Carbon dioxide is the most suited solvent for SFE of thermo labile compounds because of its favorable properties (including nontoxic and nonflammable character, a modest critical condition (31.1 °C, 7.38 MPa), high availability at low cost, high purity) and its ability to produce isolates with optimal physicochemical, biological, and therapeutic properties. The extracts obtained by SFE usually possess higher antioxidant activities than those obtained by solvent extraction probably owing to a lower degradation of active ingredients when using SFE compared with the extraction with organic solvents [35]. This emerging clean technology provides solvent free extracts and the selectivity of the supercritical CO_2 can be adjusted by varying temperature and pressure to obtain the fractions consisting of desirable a compound or mixtures.

The aim of this study was to determine the effect of antioxidant fractions obtained by SFE, from rosemary, sage, thyme and hyssop on lipid oxidation of sunflower oil, as well as to compare the antioxidant activity of the investigated extracts and the activity of synthetic antioxidants butylated hydroxyanisole (BHA) and commercial rosemary antioxidant Flavor' Plus. This study extends our previous work [36], and considers the relationship of the SFE extracts of investigated plants obtained at 35 MPa and 100 °C, and oxidative stability of sunflower oil. The antioxidant activity of the SFE extracts in preventing oxidation in food was measured in terms of its peroxide value (PV) in sunflower oil. To our knowledge, there are no previous data available in the literature on the effect of adding SFE extracts of investigated plants obtained at

35 MPa and 100 °C to oxidative stability of sunflower oil. Also, there is no data at all on determination of PV of sunflower oil with the addition of sage and hyssop extracts obtained by the SFE.

EXPERIMENTAL

Materials and methods

Materials. The sunflower oil without addition of any antioxidants was obtained from "Vital" oil and Vegetable Fat Factory (Vrbas, Serbia). BHA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Commercial rosemary antioxidant Flavor' Plus was obtained from Naturex (France).

Antioxidant fractions were isolated from dried leaves of selected herbs belonging to the Lamiaceae family: rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), thyme (*Thymus vulgaris*) and hyssop (*Hyssop officinalis*) originated from the southern Balkans region.

All other chemicals and solvents used were purchased from Merck (Darmstadt, Germany) and were of analytical grade.

Supercritical CO_2 extraction

Plant material was milled and sieved and the fraction of the average particle diameter of 0.400 mm was used for further study. The initial mass of the plant samples used for the extractions was 56.0 g for rosemary, 56.5 g for sage, 54.0 g for thyme and 55.0 g for hyssop. The fractional extraction with supercritical carbon dioxide was performed in the Autoclave Engineers Screening System previously described [37]. Foremost, the essential oil rich fraction was isolated at mild conditions (11.5 MPa; 40 °C) for 2.0-2.5 h. The extraction of antioxidant fraction followed at the pressure of 35 MPa and the temperature of 100 °C for 5.0-5.5 h [36]. The flow rate of SC-CO_2 was 0.6 kg/h for the SFE of the essential oil rich fraction, and 0.3 kg/h for the SFE of the antioxidant fraction.

HPLC Analyses of the antioxidant fractions with DAD and MS detection

According to our previous work [36], qualitative and quantitative analyses were carried out with the 1200 Agilent binary pump system (Waldbronn, Germany). For UV-Vis identification and quantification of carnosol and carnosic acid, the UV detector was monitored at 240 nm, and peak spectra were recorded between 190 and 450 nm by using a DAD. Carnosol and carnosic acid, among the main compounds present in examinated extracts, were quantified with regards to a pure standard. Their content in different plant extracts are presented in Table 1. Other compounds were characterized for their retention times (t_R), mass spectra and UV spectra and were tentatively identified based on previous data published by other authors. Some of the tentatively identified compounds in the antioxidant fractions of selected herbs obtained at 35 MPa and 100 °C were jasmonic acid, cirsimaritin, rosmanol, genkwanin, rosmadial, rosmaridiphenol and methyl-carnosate for rosemary; rosmanol, royleanonic acid, horminone, genkwanin, rosmadial, epirosmanol methyl ether, royleanone and methyl-carnosate for sage; naringenin, cirsimaritin, cirsilineol, methyl rosmarinate, *p*-cymene-2,3-diol, 3,4,3',4'-tetrahydroxy-5,5'-diisopropyl-2,2'-dimethylbiphenyl and 3,4,4'-trihydroxy-5,5'-diisopropyl-2,2'-dimethyl-3,6-biphenyl for thyme, and pinonic acid, and marrubiin for hyssop [36].

Table 1. Carnosol and carnosic acid content in different plant extracts; g/100 g of extract at 240 nm [36]

Plant extract	Carnosol	Carnosic acid
Rosemary	3.9368	4.7596
Sage	6.9729	13.7639
Thyme	-	-
Hyssop	7.3341	-

Determination of antioxidant activity by PV test

Antioxidant fractions, BHA and Flavor' Plus were dissolved separately in sunflower oil at concentrations of 200 mg/kg oil and the samples were stored in an oven maintained at 98 °C. PV on each sample was determined by the official method ISO 3960:1977 [38]. PV was expressed in milliequivalents of peroxide produced per 1 kg of the sample (meq O_2/kg). For the control, the sample without added antioxidant was used (control).

The period of PV measurement time of was in accordance with Regulation [39] (hours needed for the PV of the sample to reach maximal permitted level of 10 meq O_2/kg of oil).

The concentration of 200 mg antioxidant/kg oil was chosen according to Regulation [40], where total concentration of BHA, BHT or gallates, added separately or in combination to fats and oil (for heat-treated foodstuffs), must not exceed 200 mg/kg of the fat or oil content.

Statistical analysis

Statistical data analysis of PV test was carried out using Origin 6.0 software (OriginLab Corporation, USA). All measurements were done in triplicate and presented as mean ± standard deviation (*SD*).

RESULTS AND DISSCUSION

Extraction yields

According to our previous work [36], extraction yields (mass%) of essential oil fractions from rosemary, sage, thyme and hyssop were 0.676, 2.32, 0.754 and 0.651%, respectively. After removing the essential oil fraction which contained the main aroma compounds, SFE of antioxidant fraction commenced. Extraction yield curves are presented in Figure 2.

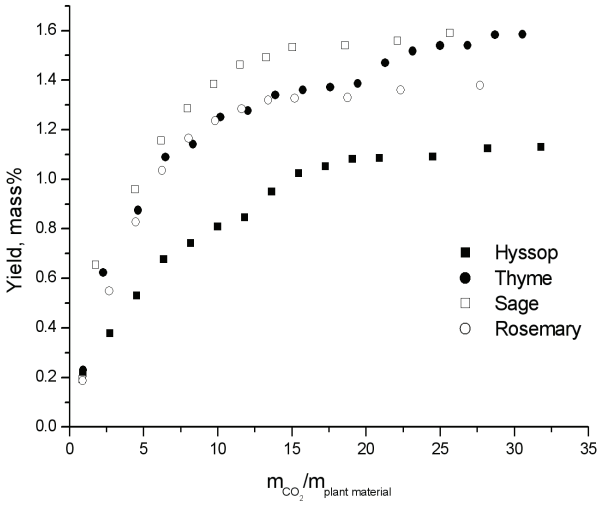

Figure 2. Yield of antioxidant fraction of rosemary, sage, thyme and hyssop as the function of specific amount of solvent (kg CO_2/ kg plant material) for SFE at 35 MPa and 100 °C.

Oxidative stability of oil with antioxidants

PV is a relatively simple test to perform and it is frequently used to investigate shelf-life or to examine the antioxidant properties of various additives. Peroxides (R-OOH) are primary reaction products formed in the initial stages of oxidation, and therefore give an indication of the progress of lipid oxidation. One of the most commonly used methods to determine PV utilizes the ability of peroxides to liberate iodine from potassium iodide. As far as our literature survey could ascertain, there were only two reports on determination of PV of sunflower oil with the addition of SFE antioxidant extracts [30,41]. Hadolin *et al.* [41] investigated the antioxidant activity of SFE rosemary extract in sunflower oil at 98 °C. The addition of rosemary extract was calculated on the content of carnosic acid in oil, and the result was 200 mg carnosic acid/kg oil. According to data presented in Table 1, it can be calculated that the concentration of carnosic acid in investigated samples of sunflower oil which contained rosemary and sage extracts were 95.2 mg carnosic acid/kg oil and 275.3 mg carnosic acid/kg oil, respectively.

The results of PV measurements as a function of time are shown in Figure 3. A control sample showed the highest formation of peroxides. The samples with synthetic antioxidant (BHA) and commercial natural antioxidant (Flavor' Plus) showed the slowest formation of peroxides after 2 h of storage at 98 °C. Rosemary antioxidant fraction had the highest antioxidant activity after 4, 6, 8, 10 and 12 h of storage at 98 °C. It was found that the rosemary extract had the antioxidant activity which was significantly higher than BHA in sunflower oil.

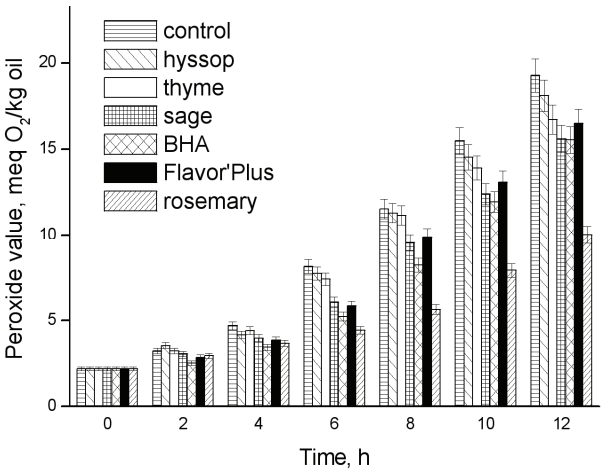

Figure 3. The effect of rosemary, sage, thyme and hyssop antioxidant fractions on the peroxide value of the sunflower oil compared to the commercial natural and synthetic antioxidants.

Significant differences between the control and the other samples in their PVs started to appear after 6 h of storage at 98 °C. PV for sunflower oil with no addition of antioxidant fraction after 6, 8, 10 and 12 h of storage at 98 °C was almost two times higher compared to the sample with added rosemary antioxidant fraction. As shown in Figure 2, the PV of sunflower oil with the addition of sage antioxidant after 6 h was 6.06 meq O_2/kg of oil, while the PV of control was 8.14 meq O_2/kg of oil. Sage antioxidant fraction showed much stronger antioxidant activity compared to the thyme and hyssop antioxidant fractions. The activity of sage antioxidant fraction was comparable to the activity of BHA, and after 12 h of storage at 98 °C its activity was equal to BHA. Thyme antioxidant fraction showed some activity, and after 12 h of storage at 98 °C its activity was similar to Flavor' Plus. The hyssop antioxidant fraction was weakest and its activity was close to the control.

On the basis of PV measurements the order of antioxidative activity after 12 h of storage at 98 °C was: rosemary extract > BHA >sage extract > Flavor' Plus > thyme extract > hyssop extract. Better activities

were obtained with rosemary and sage extracts which were comparable to BHA and Flavor' Plus. We concluded that the presence of carnosic acid which is a lipophilic antioxidant significantly contributed to the antioxidant activities of rosemary and sage extracts. This is in accordance with the results of Braida *et al.* [42], Trojakova *et al.* [43], and Ying *et al.* [44], who had reported that there were high correlations between the carnosic acid content and the protection factor in high linoleic acid content oil.

Among rosemary's and sage's antioxidant compounds, carnosic acid is believed to possess the highest antioxidant activity [45-47]. On the other hand, since sage extract included higher carnosol and carnosic acid content than rosemary extract (Table 1), there may be other unidentified compounds in the rosemary extract that provide additive or synergistic antioxidant effects which would explain greater activity in the case of rosemary extract. For example, among tentatively identified compounds in the rosemary extract methyl carnosate was a better antioxidant than carnosic acid in bulk oils and oil-in-water emulsions [48]. Also, investigated extracts may show positive or negative synergistic effects with components from sunflower oil and different thermal stability. Hadolin *et al.* [41] obtained rosemary extract by successive extraction with conventional solvents and SC-CO_2 extraction. The authors [41] reported that PV of sunflower oil with no addition of antioxidant after 8 h of storage at 98 °C was two times higher (32 meq O_2/kg of oil) compared to the sample with added rosemary extract (16 meq O_2/kg of oil), while in the present study PV of sunflower oil after 8 h was 11.54 meq O_2/kg of oil (control) and 5.64 meq O_2/kg of oil with rosemary extract.

The outcome of the number of assays which used to evaluate the antioxidant properties of natural antioxidants varied from test to test. The results obtained in the present study were not in agreement with our previous reports [36], using DPPH radical assay and hydroxyl radical assay to evaluate the antioxidant capacity of investigated antioxidant fractions because thyme extract showed the highest antioxidative activity among the examined extracts in DPPH assay, while sage extract showed the highest antioxidative activity among the examined extracts in hydroxyl radical assay. Dapkevicius *et al.* [31] reported that rosemary, sage and thyme extracts obtained by SFE possessed a high antioxidant activity in β-carotene bleaching test, while hyssop extracts showed a low antioxidant activity which is in accordance with the results obtained in PV test.

CONCLUSIONS

A high pressure extraction is a very appropriate technique for isolating natural thermolabile substances. The product does not contain residual organic solvents as in conventional extraction processes, which makes these products suitable for use in food, cosmetic and pharmaceutical industry. The efficiency of rosemary, sage, thyme and hyssop antioxidant extracts was performed by measuring the peroxide value. The best results were obtained with rosemary and sage extracts which were more efficient compared to other extracts and their antioxidant activity was comparable to the activities of BHA and Flavor' Plus. The obtained results indicated that the presence of carnosic acid significantly contributed to antioxidant activities of rosemary and sage extracts. Further research is needed in order to obtain more reliable results on the determination of chemical composition of antioxidant fractions, especially in the case of rosemary, and to elucidate the compounds that contributed to the strong antioxidant activity of rosemary extract. Lipid oxidation products make the oil unfit for human health, therefore in order to minimize the oxidation phenomenon, some antioxidants should be added to increase the storage and shelf-life of oils and oil products. Results of the present study demonstrate positive effects of adding antioxidant fractions from rosemary and sage on retarding lipid oxidation of sunflower oil.

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NAUČNI RAD

OKSIDATIVNA STABILIZACIJA SUNCOKRETOVOG ULJA SA ANTIOKSIDATIVNIM FRAKCIJAMA IZ ODABRANIH BILJAKA FAMILIJE LAMIACEAE

U radu je ispitivan uticaj antioksidativnih frakcija iz odabranih biljaka familije Lamiaceae: ruzmarina (Rosmarinus officinalis), žalfije (Salvia officinalis), timijana (Thymus vulgaris) i izopa (Hyssop officinalis) na oksidativnu stabilnost suncokretovog ulja. Antioksidativne frakcije su izolovane primenom frakcione ekstrakcije sa natkritičnim ugljenik(IV)-oksidom na pritisku od 35 MPa i temperaturi od 100 °C. Ispitivane frakcije su dodate suncokretovom ulju u koncentraciji od 200 mg/kg ulja i uzorci su čuvani na temperaturi od 98 °C. Antioksidativna aktivnost ekstrakata određivana je merenjem peroksidnog broja. Antioksidativna aktivnost ekstrakata bila je upoređivana sa aktivnošću butilovanog hidroksianizola (BHA) i komercijalnog ruzmarinskog ekstrakta Flavor' Plus. Svi ispitivani ekstrakti pokazali su se kao inhibitori lipidne peroksidacije suncokretovog ulja. Najbolju antioksidativnu aktivnost među ispitivanim ekstraktima pokazao je ekstrakt ruzmarina. Na osnovu vrednosti peroksidnog broja antioksidativna aktivnost ispitivanih biljnih ekstrakata posle 12 h čuvanja na 98 °C bila je sledeća: ekstrakt ruzmarina > BHA > ekstrakt žalfije > > Flavor' Plus > ekstrakt timijana > ekstrakt izopa.

Ključne reči: peroksidni broj; ruzmarin; žalfija; timijan; izop; natkritična ekstrakcija.