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Enrichment of olive oil with sage (*Salvia officinalis*) leaf essential oil extracted by ultrasound pretreatment followed by hydrodistillation to increase its shelf life

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Abstract

Olive oil (OO) is a valuable food product, compared with other seed oils, for its health benefits, sensory attributes and higher economic value. The demand for natural antioxidants has recently grown due to the potential toxicity and carcinogenicity of synthetic antioxidants that are utilized in food industry. This study aimed to investigate the oxidative stability of OO enriched with sage (*Salvia officinalis*) essential oil (EO). The sage EO was extracted from the leaves by ultrasound (US) as a pretreatment followed by hyrodistillation (HD). The effects of US power level (60 and 90 W) and time (10, 20 and 30 mins) on the EO yield, total extraction time and quality characteristics were studied. Compared to the HD extraction, the US pretreatments before HD shortened the total extraction time to ≤ 54 mins, with the same extraction yield, instead of 240 mins with HD. Various EO samples extracted by these US pretreatments followed by HD (US+HD) were analyzed for their chemical compositions by GC–MS and compared with that of EO obtained by HD. Compositional analysis revealed that the most prominent oxygenated monoterpenes of EO samples were camphor, α-thujone, 1,8-cineole, β-thujone, bornyl acetate and *endo*-borneol. The EO extracted by US pretreatment at 60 W for 10 mins followed by HD for 34 mins was characterized by the highest quality index [(α- + β-thujone)/camphor ratio] and higher levels of antioxidant constituents (α-pinene and camphene). The sage EO obtained by this technique was selected to enrich OO at 200, 400 and 600 mg/Kg of OO. Quality criteria of the OO such as acidity, peroxide value, conjugated dienes and trienes besides chlorophyll and carotenoid contents and fatty acids composition were measured. Results revealed that the addition of sage EO at 200 mg/Kg of OO increased the oxidative stability of OO measured by Rancimat by 36% instead of 21% by using the synthetic antioxidant butylated hydroxytoluene (BHT) at the same level. Moreover, the sensory attributes of the enriched OO were maintained. From this study, it can be concluded that sage EO can be used to increase the shelf life of OO.

1. Introduction

The oxidation of oil produces unpleasant odour and flavour. Antioxidants are often added to edible oils to retard oxidation during storage. Nowadays, studies have been focused on the replacement of synthetic antioxidants with natural ones due to possible health risks associated with synthetic antioxidants. A large assortment of olive oil enriched with spices and EOs has been used for seasoning many foodstuffs (Benkhoud *et al*., 2021). Some of these EOs have antioxidant activity such as oregano, rosemary and thyme (Barreca *et al*., 2021). Incorporation of synthetic antioxidants into olive oil is prohibited (Codex Alimentarius, 2021).

Salvia officinalis L. belongs to the Lamiaceae family. *Salvia officinalis* is also known by other names such as Dalmatian sage, sage and common sage. The essential oil (EO) obtained from the leaves of sage has been used as food flavouring agent worldwide (Vosoughi *et al*., 2018). Recently, the treatment of ultrasound techniques for the extraction of EO has been reported. The ultrasonic intensity and time are the main factors that affect the efficiency of the ultrasound process. This ultrasound treatment technique requires a short time for extraction compared to the conventional hydrodistillation (HD) technique (Chen *et al*., 2020). Additionally, this ultrasound treatment technique has high extraction efficiency of various components including thermally

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labile components. Therefore, the aim of this work was focused on the extraction of EO from the leaves of sage using ultrasound pretreatment (US) followed by HD, evaluate its antioxidative effect in olive oil and determine the sensory acceptability of the enriched olive oil.

2. Materials and methods

2.1 Materials

Extra-virgin olive oil (3 kg) was obtained from an olive oil mill, Agriculture Research Center, Giza, Egypt. Sage (*Salvia officinalis* L.) dried leaves (2 kg) were procured from Harraz Company for Medicinal Plants, Cairo, Egypt. Butylated hydroxytoluene (BHT), standard mixtures of fatty acid methyl esters (FAME) (C_8-C_{22}) and homologous series of n-alkanes (C_8-C_{20}) for the determination of kovats indices were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Ethyl alcohol, methyl alcohol, potassium hydroxide, chloroform, sodium thiosulfate, sodium sulphate anhydrous, potassium iodide, glacial acetic acid, isooctane, cyclohexane and sulphuric acid were purchased from Merck Co. (Darmstadt, Germany).

2.2 Extraction of essential oil from sage

2.2.1 Hydrodistillation

Sage dried leaves (50 g) were ground into fine powder. The HD process was carried out using a Clevenger apparatus for 4 hrs according to Charchari and Abdelli (2014). The extraction yield (%) was calculated as the volume of EO (mL) per 100 g of dried leaves. The hydrodistillation was repeated three times and the extracted EO samples were dried with anhydrous Na_2SO_4 and kept in amber vials at 4°C prior to analysis and use.

2.2.2 Ultrasound-assisted hydrodistillation extraction of sage essential oil

Approximately 50 g of the leaf powder of sage was mixed with 600 mL distilled water. The mixture was sonicated using Fisher Sonic Dismemberator (Model 300, 50 Hz, USA) at room temperature (25°C). Extraction was performed at 60 W for 10, 20 and 30 mins (Techniques A, B and C, respectively) and at 90 W for 10, 20 and 30 mins (Techniques D, E and F, respectively). After each US treatment, the resulting mixture was diluted with an additional 350 mL of distilled water and subjected to HD as reported previously. Extraction time during HD after US pretreatment was extended until no more EO was obtained. The extraction time was recorded and the yield was calculated. Each US+HD treatment was carried out in triplicate.

2.3 Analysis of the chemical composition of sage essential oils by GC-MS

The GC–MS analysis of each EO was performed according to Morsy and Hammad (2021). Identification of the volatile components of the EO was based on a comparison of their Kovats indices (RI) and mass spectra with Wiley spectral library collection and NIST library. The quantification of the volatile constituents was performed with relative area percentage. The EO with the unique quality was selected for the enrichment of olive oil.

2.4 Preparation of olive oil samples enriched with sage essential oil

The addition of the sage EO with the highest quality index and high levels of antioxidant constituents to the olive oil (50 g) was carried out at 200, 400 and 600 mg/kg. Olive oil sample without EO was used as a control. The enrichment was carried out in triplicate for each concentration. Oil samples were kept in dark glass bottles at 5°C till analysis.

2.5 Determination of quality indices of olive oil

Acidity (% oleic acid), peroxide value (meq O_2/kg oil) and conjugated diene and triene, (specific extinction of 1% solution of oil in isooctane measured with UV-Vis spectrophotometer at 232 and 270 nm (K_{232}, K_{270})), were determined according to Regulation EEC 2568/91 of the European Union Commission (1991). Chlorophyll (pheophytin) and carotenoids (lutein) contents (mg pigment/kg oil) were determined spectrophotometrically in cyclohexane at 670 nm and 470 nm, respectively (Martakos *et al*., 2020).

2.6 Analysis of fatty acids profile of olive oil by GC-MS

For analyzing fatty acids with GC it is necessary to convert them into FAME derivatives for greater volatility (Milinsk *et al*., 2008; Visentainer *et al*., 2014). The FAMEs of olive oil samples were prepared by the transesterification of oil in hexane with sodium methylate according to Regulation EEC 2568/91 of the European Union Commission (1991). The analysis of FAMEs was carried out using an Agilent 6890 series gas chromatograph equipped with a DB23 (60 m × 0.32 mm × 0.25 µm capillary column (Agilent Technologies Inc., CA, USA). Nitrogen flow rate was 1.6 mL/min. The injection temperature was 250°C and flame ionization detector (FID) was set at 270°C. The split ratio was 1:50. The GC oven temperature program was set as follows: 150°C for 1 min, 10°C/min to 170°C (5 mins hold), 5°C/min to 220°C (3 mins hold) for a total runtime of 21 mins. The standards of FAMEs were used for identification. The relative area percentages of the

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identified fatty acids were calculated from the total peak areas of FAMEs.

2.7 Oxidative stability

Oxidative stability of oils is defined as the resistance to oxidation during processing and storage (Guillen and Cabo, 2002). It is considered the most important quality parameter of edible oils (Maszewska *et al*., 2018). The oxidative stability of olive oil samples was measured according to American Oil Chemists' Society (AOCS) (1992) using Rancimat 679 apparatus (Metrohm, Herisau, Switzerland). The Rancimat method is officially recommended for the assessment of oxidative stability of edible oils in a relatively short time, at high temperatures and intensive aeration. The progressive increase of electric conductivity of deionized water was monitored while air (20 L/h) was bubbled into each heated oil sample (5 g, 110°C). The volatile oxidation products were stripped from the oil and dissolved in the deionized water. The abrupt increase in the conductivity of water characterizes the induction period (Farhoosh, 2007). The induction period was determined as the inflection point of the conductivity curve (mS/cm) of the water versus time (hrs) (Martín *et al*., 2014). BHT was used at 200 mg/Kg for comparison. Protection factor (PF) was calculated as described by Mendez-Encinas *et al*. (2020). It expresses the ratio of the induction period of oil in the presence of the antioxidant (test material) to the induction period of oil without any additive.

2.8 Sensory evaluation of olive oil samples enriched with sage essential oil

Sensory assessment of oil samples was performed according to Asensio *et al*. (2013). Fresh white bread (slices 3 cm × 3 cm × 1 cm) was used as a carrier and olive oil samples (either control or enriched with sage essential oil) were added at a constant ratio (Moldão-Martins *et al*., 2004). Ten panelists (6 males and 4 females) with an age range of 30-45 years, were asked to evaluate each sample separately. A 9-point numeric scale, ranging from dislike extremely (1) to like extremely (9) was used to rank the samples according to their odour, taste (fruity, bitterness and pungency) and acceptability scores.

2.9 Statistical analyses

Extraction yield of EO, quality indices, oxidative stability and sensory analysis of olive oil samples were triplicated and the results were expressed as mean±standard deviation (SD). Analysis of variance (one-way ANOVA) and Tukey's test at $P < 0.05$ were carried out using XLSTAT software.

3. Results and discussion

3.1 Effect of the extraction conditions on total extraction time and essential oil yield

The yield of EO extracted with HD was found to be 0.8±0.015% (Table 1). It needed 240 mins for maximum extraction of EO, confirming what was reported by Charchari and Abdelli (2014). The US pretreatments reduced HD time to ≤ 34 mins only without a significant decrease in the EO yield (0.8%), regardless of the ultrasonic intensity used. Furthermore, the US pretreatments (10-30 mins) decreased total extraction time to ≤ 55 mins.

3.2 Chemical profile of sage essential oil

GC-MS analysis indicated that oxygenated monoterpenes were the predominant compounds in sage EO (Table 1). The main components in the oxygenated monoterpenes are camphor, α-thujone, 1,8-cineole, β-thujone, bornyl acetate and *endo*-borneol. The chemical profile of sage EO is consistent with the data reported by Chrysargyris *et al*. (2021). High quality sage EO is characterized by α- + β-thujone up to 50% of the EO constituents, and the percentage of camphor ≥ 20% (Putievsky *et al*., 1992). Therefore, the (α- + β-thujone)/ camphor ratio has to be approximately 2.5 for good quality sage EO. In the present study, the highest ratio of 1.73 was determined for EO obtained by US+HD (Technique A) instead of 1.2 by the conventional HD method. The EO extracted by Technique A was characterized by higher levels of antioxidant constituents (camphene and α-pinene) compared to that of EO obtained by only HD or other US+HD investigated techniques. Diepicedrene 1 oxide, calarene epoxide and isoamaderdrene epoxide were identified in the EO extracted by HD without US pretreatment due to thermal degradation during the long extraction time. According to results in Table 1, sage EO obtained by technique A was selected, for its high quality and high level of antioxidant constituents. Figure 1 shows the chromatograms of sage EO obtained by HD and that obtained by US+HD (technique A).

3.3 Quality indices of olive oil

The quality characteristics of the olive oil sample showed that the acidity (%), peroxide value (meq O_2/Kg oil) and specific coefficients of extinction at 232 and 270 nm were: 0.67±0.06, 6.12±0.15, 1.83±0.01 and 0.15±0.01, respectively. The maximum limits of these parameters are ≤ 0.8%, ≤ 20 meq O_2/Kg, ≤ 2.5 and ≤ 0.22, respectively, for extra virgin olive oil according to Regulation EEC 2568/91 of the European Union Commission (1991). In the present study, total chlorophylls and carotenoid contents (mg/Kg) were

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Table 1. Volatile components relative area percentage (%)[1] of sage EO obtained by different techniques

Identified Compounds	RI[2]	HP5MS LRI[3]	HD[4]	A[5]	B[5]	C[5]	D[5]	E[5]	F[5]
Monoterpene Hydrocarbons									
Tricyclene	927.49	927	0.20	-	0.19	-	0.25	-	0.21
α-Pinene	936.25	939	4.24	5.60	5.19	5.01	6.02	4.43	5.46
Camphene	952.99	954	4.41	6.14	5.58	5.66	6.72	5.29	5.83
β-Pinene	980.88	979	1.21	0.87	0.73	0.86	1.07	0.96	0.86
β-Myrcene	992.83	991	0.23	0.72	0.18	0.48	0.54	0.60	0.20
ρ-Cymene	1019.6	1025	0.14	0.72	-	0.56	0.54	0.59	-
D-Limonene	1031.1	1032	1.30	1.73	1.39	1.58	2.12	1.96	1.37
Oxygenated Monoterpenes									
1,8-Cineole	1034.4	1035	12.31	10.14	9.04	9.72	11.04	10.58	10.86
α-Linalool	1107.3	1102	0.26	0.42	-	0.52	0.28	0.40	0.09
α-Thujone	1114.9	1117	22.02	27.30	30.61	26.20	26.24	26.48	29.82
β-Thujone	1125.3	1121	6.71	10.16	8.37	10.70	9.23	10.24	8.09
Camphor	1157.1	1146	23.84	21.63	24.88	23.08	23.43	24.40	25.67
Isothujol	1177	1166	0.24	0.29	0.12	0.39	0.26	0.33	-
endo-Borneol	1180.3	1169	2.31	1.79	1.67	2.57	2.06	2.39	1.92
Terpinen-4-ol	1188.9	1179	0.23	0.30	0.12	0.34	0.22	0.31	0.13
α-Terpineol	1206.7	1189	0.23	0.26	-	-	-	0.22	0.09
Bornyl acetate	1287.9	1289	2.61	3.02	2.47	3.72	2.72	3.22	2.85
Myrtenyl acetate	1294.8	1297	0.24	0.50	0.25	0.50	0.31	0.39	0.22
Sesquiterpene Hydrocarbons									
Trans-β-Caryophyllene	1416.7	1420	2.39	0.67	0.90	0.73	0.67	0.69	0.63
α-Humulene	1453.7	1455	4.77	1.92	2.59	1.92	1.70	1.75	1.75
Oxygenated Sesquiterpenes									
Caryophyllene oxide	1583.1	1583	0.38	0.27	0.24	0.26	0.22	0.24	0.18
Viridiflorol	1596.2	1593	5.67	3.34	3.47	2.98	2.66	2.75	2.32
Ledene oxide	1601.1	1600	0.32	0.34	0.26	0.30	0.25	0.27	0.18
Humulene 1,2 epoxide	1612.3	1608	1.62	1.35	1.29	1.26	1.08	1.14	0.98
Diepicedrene 1 oxide	1634.3	1630	0.08	-	-	-	-	-	-
Calarene epoxide	1637.3	1641	0.60	-	-	-	-	-	-
Isoamaderdrene epoxide	1661.7	1655	0.10	-	-	-	-	-	-
Diterpene Hydrocarbons									
Isopimaradiene	1907.1	1905	0.10	0.52	0.36	0.41	0.36	0.37	0.21
Monoterpene Hydrocarbons			11.73	15.78	13.26	14.15	17.26	13.83	13.93
Oxygenated Monoterpenes			71.00	75.81	77.53	77.74	75.79	78.96	79.74
Sesquiterpene Hydrocarbons			7.16	2.59	3.49	2.65	2.37	2.44	2.38
Oxygenated Sesquiterpenes			8.77	5.30	5.26	4.80	4.21	4.40	3.66
Diterpene Hydrocarbons			0.10	0.52	0.36	0.41	0.36	0.37	0.21
Total oxygenated compounds (%)			79.87	81.11	82.79	82.54	80.00	83.36	83.40
Total non-oxygenated compounds (%)			18.99	18.89	17.11	17.21	19.99	16.64	16.52
Total identified components			98.76	100.00	99.90	99.75	99.99	100.00	99.92
Ratio (α-+β-thujone)/camphor			1.20	1.73	1.57	1.60	1.51	1.50	1.48
% Yield (v/w)[6]			0.80± 0.015[a]	0.80± 0.01[a]	0.80± 0.017[a]	0.80± 0.015[a]	0.80± 0.01[a]	0.80± 0.017[a]	0.80± 0.015[a]
Ultrasonic Extraction time (min)			-	10	20	30	10	20	30
HD Extraction time (min)			240	34	25	25	25	14	15
Total Extraction time (min)			240	44	45	55	35	34	45

[1] Relative area percentage

[2] Retention indices

[3] LRI, Retention indices from the literature on HP-5MS column

[4] Hydrodistillation

[5] Ultrasonic assisted extraction (US) prior to HD. US at 60 W for 10, 20 and 30 mins (Techniques A, B and C, respectively) and at 90 W for 10, 20 and 30 mins (Techniques D, E and F, respectively).

[6] Values are presented as mean±SD. Values with different superscripts within the same row are statistically significantly different ($P < 0.05$).

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found to be 2.41±0.11 and 1.68±0.01, respectively. These results are consistent with those obtained by Asensio *et al*. (2013) for fresh olive oil samples. Both pigments enhance the oxidative stability of olive oil (Keramat *et al*., 2016).

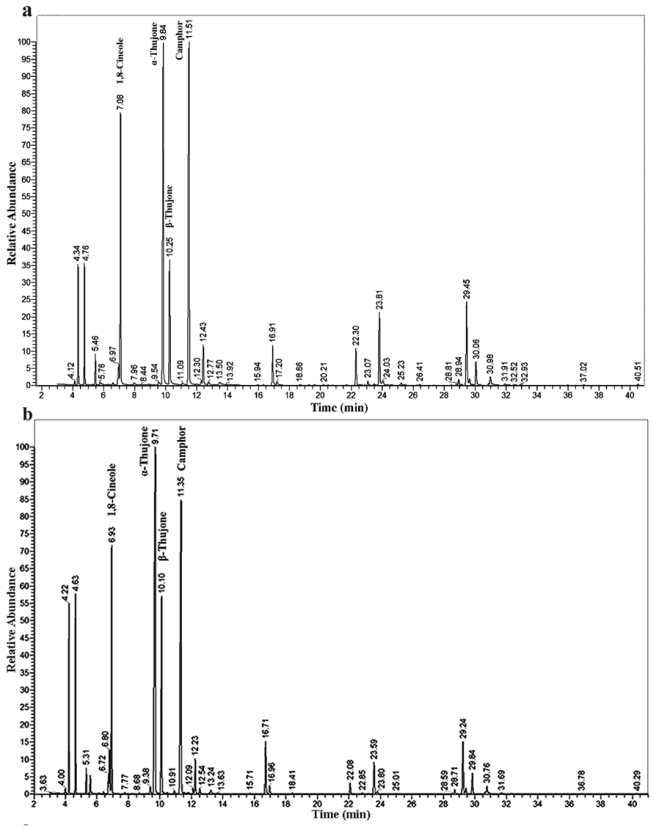

Figure 1. GC-MS chromatograms of sage essential oil samples obtained by: a) Conventional hydrodistillation (HD) method and b) Ultrasound pretreatment (60 W, 10 mins) followed by HD for 34 mins.

3.4 Fatty acids profile of olive oil

The fatty acid composition of the olive oil is shown in Table 2 and Figure 2. Oleic acid (C18:1) was the predominant fatty acid (70.21%), followed by palmitic acid (C16:0) (15.22%), linoleic acid (C18:2) (8.93%), stearic acid (C18:0) (2.31%), palmitoleic acid (C16:1) (1.24%) and linolenic acid (C18:3) (0.99%). These results are in accordance with the chemical profile of olive oil as reported by Borges *et al*. (2017). They studied the fatty acid composition of extra virgin olive oil from two regions in Brazil and nine regions in Spain and found that the ranges of the fatty acid contents were: C18:1 (63.12–79.68%), C16:0 (11.99–19.55%), C18:2 (6.56–14.76%), C18:0 (1.44–2.13%), C16:1 (0.58–3.16%) and C18:3 (0.46–0.69%). According to Regulation EEC 2568/91 of the European Union Commission (1991), C18:3 of olive oil has to be ≤ 1%. In the present study, total monounsaturated fatty acids (MUFA, 71.87%) were the largest group, followed by saturated fatty acids (SFA, 18.21%), and polyunsaturated fatty acids (PUFA, 9.92%). The ratios between Oleic acid/Linoleic acid, MUFA/PUFA and PUFA/SFA were 7.86, 7.24 and 0.54%, respectively. Similar results were reported by Fagundes *et al*. (2020). The high MUFA/PUFA ratio of olive oil confers its oxidative stability as reported by Jimenez-Lopez *et al*. (2020).

Table 2. Fatty acid composition of olive oil

Identified fatty acids		Relative Area %
Myristic acid	(C14:0)	0.01
Pentadecanoic acid	(C15:0)	0.02
Palmitic acid	(C16:0)	15.22
Palmitoleic acid	(C16:1)	1.24
Margaric acid	(C17:0)	0.06
Heptadecenoic acid	(C17:1)	0.10
Stearic acid	(C18:0)	2.31
Oleic acid	(C18:1)	70.21
Linoleic acid	(C18:2)	8.93
Linolenic acid	(C18:3)	0.99
Arachidic acid	(C20:0)	0.46
Gadeloic acid	(C20:1)	0.32
Behenic acid	(C22:0)	0.13
Total area %		100.00
Saturated fatty acids (∑ SFA)		18.21
Monounsaturated fatty acids (∑ MUFA)		71.87
Polyunsaturated fatty acids (∑ PUFA)		9.92
Oleic acid / linoleic acid		7.86
MUFA/PUFA		7.24
PUFA/SFA		0.54

The values refer to a single determination.

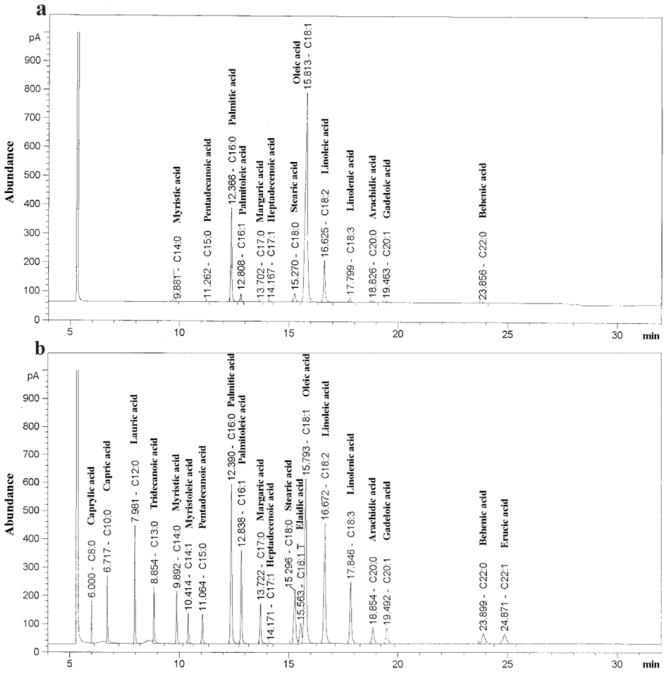

Figure 2. GC chromatograms of a) fatty acid methyl esters from olive oil sample, b) Standard fatty acid methyl esters.

3.5 Oxidative stability

The degree of resistance provided by the oil to oxidative deterioration is referred to as oxidative stability (Mishra *et al*. 2021). The induction period of the olive oil (control) was found to be 16.2±0.1 hrs (Table 3). This

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indicates the quality and oxidative status of the oil (Sousa *et al*., 2015).

Olive oil enriched with sage EO (technique A) at 200, 400 and 600 mg/kg showed higher resistance to oxidation compared with their corresponding control by 36, 6 and 4%, respectively. 1,8-cineole and camphor, camphene and α-pinene in the EO have the ability to eliminate the radical chain reaction (Quintans-Júnior *et al*., 2013; Noacco *et al*., 2018; Wang *et al*., 2019). In the current study, higher concentrations of the investigated EO restrained significantly ($P < 0.05$) its antioxidant activity. The oxidative stability of olive oil (measured by Rancimat at 110°C) decreased significantly with the increase of the incorporation level of lemon verbena EO (Cherif *et al*., 2021). They attributed this negative effect to the possible prooxidant activity of some essential oil constituents. The addition of BHT to the olive oil sample at 200 mg/kg, for comparison, extended the induction period of oil by only 21%. The antioxidant property of the EO could be attributed to the synergistic effect of its constituents rather than a single compound.

Table 3. Oxidative stability of olive oil enriched with different levels of sage EO.

Samples	Induction period (hrs)	Protection factor*
Olive oil (Control)	16.20±0.10^{d}	1.00
Olive oil + sage EO at 200 mg/Kg	22.10±0.20^{a}	1.36
Olive oil + sage EO at 400 mg/Kg	17.23±0.15^{c}	1.06
Olive oil + sage EO at 600 mg/Kg	16.90±0.20^{c}	1.04
Olive oil + BHT at 200 mg/Kg	19.60±0.20^{b}	1.21

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different ($P < 0.05$).
*The protection factor is the ratio of the induction period of sample to that of control.

3.6 Sensory evaluation of olive oil enriched with sage essential oil

Enriched olive oil samples showed significantly ($P < 0.05$) the same intensity of fruity taste (Table 4). In terms of sensorial attributes, all olive oil samples enriched with EOs (except the sample at a ratio of 200 mg/Kg) exhibited no better attributes than the control. The olive oil sample with EO at a ratio of 200 mg/kg showed marginally higher scores than control only for bitterness and pungency (Table 4). Enrichment with EOs at a ratio of 200 mg/kg preserved the sensory attributes of olive oil. However, enrichment with a higher ratio of EOs did not preserve these attributes but imparted undesirable sensory characteristics.

Moldão-Martins *et al*. (2004) found that the taste of olive oil flavoured with EOs mixture (0.008% thyme + 0.002% mentha) recorded the highest acceptability while flavouring with mentha EO at 0.2% showed the lowest acceptance. The addition of oregano EO to olive oil at 0.05% (w/w) protected the sensory properties (fruity, pungency and bitterness) of olive oil and decreased the oxidation process (Asensio *et al*., 2013). Enriching virgin olive oil with *Rosmarinus officinalis* EO at 1000 ppm increased its oxidative stability three times (Keramat *et al*., 2016).

4. Conclusion

Using US as a pretreatment followed by HD reduced the extraction time of sage EO by 82%. Quality index of EO extracted with the US+HD combined technique, measured by (α- + β-thujone)/camphor ratio, was unique. Enriching olive oil with sage EO at a ratio of 200 mg/kg provides significantly higher antioxidant activity compared to BHT. This level of enrichment preserved the sensory attributes of olive oil.

Conflict of interest

The authors declare no conflict of interest.

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Table 4. Panellists' acceptance of olive oil samples enriched with sage EO at different levels

Samples	Odour	Taste			Overall acceptability
		Fruity	Bitterness	Pungency	
Olive oil (Control)	8.6±0.55^{a}	7.8±0.45^{a}	3.18±0.46^{c}	3.0±0.35^{c}	8.2±0.84^{a}
Olive oil enriched with sage EO at					
200 mg/kg	8±1.00^{ab}	7.8±0.45^{a}	3.9±0.22^{bc}	3.5±1.06^{c}	8.2±1.00^{a}
400 mg/kg	7±1.00^{b}	7.8±0.84^{a}	4.3±0.45^{b}	4.8±0.84^{b}	7.7±0.45^{ab}
600 mg/kg	7±1.00^{b}	7.8±0.45^{a}	5.2±0.84^{a}	5.7±0.45^{a}	6.9±0.22^{b}

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different ($P < 0.05$).

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