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Antioxidant activity of rosemary essential oil fractions obtained by molecular distillation and their effect on oxidative stability of sunflower oil

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ABSTRACT

The objective of this study was to evaluate the antioxidant activity of rosemary essential oil fractions obtained by molecular distillation (MD) and investigate their effect on the oxidative stability of sunflower oil. MD fractions were prepared in a series of low-pressure stages where rosemary essential oil was the first feed. Subsequently, a distillate (D1) and residue (R1) were obtained and the residue fraction from the previous stage used as the feed for the next. The residue fractions had the largest capacity to capture free radicals, and the lowest peroxide values, conjugated dienes and conjugated trienes. The antioxidant activity of the fractions was due to oxygenated monoterpenes, specifically α-terpineol and *cis*-sabinene hydrate. Oxidative stability results showed the residues (R1 and R4) and butylated hydroxytoluene had greater antioxidant activity than either the distillate fractions or original rosemary essential oil. The residue fractions obtained by short path MD of rosemary essential oil could be used as a natural antioxidants by the food industry.

1. Introduction

Conventional sunflower oil (SO) is the most frequently consumed oil in Argentina. However, SO is susceptible to oxidation because it contains large amounts of unsaturated fatty acids, particularly polyunsaturated fatty acids, such as linoleic acid (18:2 ω-6). Lipid oxidation produces rancid odors, unpleasant flavors, and discoloration. It also decreases the nutritional quality and safety of foods due to secondary oxidation products that have harmful effects on human health (Lercker & Rodriguez-Estrada, 2002).

Natural and synthetic antioxidants are added to edible oils to delay oxidative deterioration, thereby maintaining the quality and prolonging the shelf-life of food product. The use of natural compounds as additives is in increasing demand. Rosemary (*Rosmarinus officinalis* L.) essential oil (REO) is considered a natural antioxidant, but the antioxidant compounds present have not yet been established fully.

REO composition is affected by various factors, such as weather, soil humidity, extraction method, distance between plants, harvest time, and drying method. Therefore, researchers have sought to obtain information about the yield, composition, and chemical properties of REO following a variety of extraction methods, harvesting times, and plant parts (Bousbia et al., 2009; Elamrani, Zrira, & Benjilali, 2000; Peter, 2004; Socaci, Tofana, Socaciu, Varban, & Muste, 2007; Szumny, Figiel, Gutiérrez-Ortíz, & Carbonell-Barrachina, 2010). REO chemical composition differed significantly but the major chemical constituents are α-pinene, 1,8-cineole, camphor, myrcene, and camphene (Elanrami et al., 2000; Flamini, Cioni, Morelli, Macchia, & Ceccarini, 2002; Romero Márquez, 2004; Varela et al., 2009).

Several studies have reported the antioxidant activity of REO (Beretta, Artali, Maffei Facino, & Gelmini, 2011; Bozin, Mimica-Dukic, Samojlik, & Jovin, 2007; Kadri et al., 2011; Ojeda-Sana, Van Baren, Elechosa, & Juárez, 2013; Sacchetti et al., 2005). Some authors reported that the antioxidant activity was related to the presence of compounds, such as verbenone and borneol (Sacchetti et al., 2005), but others indicate that constituents, like oxygenated monoterpenes and sesquiterpene hydrocarbons (Bozin et al., 2007), alcoholic ethers and phenolic compounds (Beretta et al., 2011), 1,8-cineol, α-pinene, β-pinene, α-thujene, trans-caryophyllene, β-thujone, borneol, and camphor (Kadri et al., 2011), or myrcene (Ojeda Sana et al., 2013), were responsible for these characteristics.

Molecular distillation (MD) is a separation technique often used to purify thermolabile substances and low volatility compounds (Pramparo, Prizzon, & Martinello, 2005; Fregolente et al., 2007; Pramparo, Leone, & Martinello, 2008; Shao, Jiang, & Ying, 2007).

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However, few studies have used MD to separate essential oil fractions.

In a previous study, MD was used to concentrate methyl chavicol from basil essential oil and the conditions were optimized by response surface methodology. The results showed that it is possible to increase the concentration of methyl chavicol from 83.81 to 89.79% (Martins et al., 2012). Borgarello, Mezza, Soltermann, and Pramparo (2014) separated fractions from oregano essential oil by MD with greater antioxidant activity. Free radical scavenging capacity (RSC) was increased in residue fractions with higher concentrations of thymol and carvacrol, terpinen-4-ol and γ-terpinene (Olmedo, Nepote, & Grosso, 2014). Other research has reported the antioxidant properties of aguaribay raw oil and its fractions prepared by vacuum distillation. Residues with the most terpinen-4-ol and germacrene D had the greatest antioxidant activity (Guala, Elder, Perez, & Chiesa, 2009).

REO has separated and concentrated previously using a three-stage MD process to produce residues with the greatest antioxidant activity possible (Mezza, Borgarello, Daguero, & Pramparo, 2013). However, it was not demonstrated clearly which compounds were responsible for these antioxidant properties. The current study focused on preparation of REO fractions by short path MD using high-pressure separation, and evaluated antioxidant activity in SO to identify the compounds responsible for the protective effect in this food product.

2. Materials and methods

2.1. Materials

REO was donated by Platario SA (Buenos Aires, Argentina). The essential oil was derived from *Rosmarinus officinalis* L. that had been harvested in August 2011. The REO was produced in Barreal, San Juan, Argentina, located at 31° 40′ S latitude and 69° 29′ W longitude, at 1650 m above sea level. The essential oil was obtained by hydrodistillation by Platario SA. The essential oil was dried over anhydrous sodium sulfate, preserved in sealed flasks, and stored at 4–6 °C until analysis.

Refined SO (Natura brand, from Aceitera General, Córdoba, Argentina) was used for the storage assays to evaluate the antioxidant properties of the molecularly distilled fractions, based on deterioration using lipid oxidation indicators.

2.2. Methods

2.2.1. MD process description

The MD was performed in four stages using a DCC4 falling film distiller (Ingeniería Bernoulli SA, Buenos Aires, Argentina) equipped with a 0.04 m^2 evaporation surface, a condensing surface of 0.02 m^2, and variable speed rotating rollers. In the first stage, REO was used and two fractions, a distillate (D1) and residue (R1), were obtained. In subsequent steps (2–4), residue fractions obtained from the previous stage was used as the feed.

MD operating conditions are presented in Table 1. For all stages, the condenser temperature was set at −2.1 °C; the feed was kept at room temperature; the evaporation temperature was maintained at 26 ± 1 °C; the feed flow was around 1.10 ± 0.05 mL/min; and the rotor speed was kept constant at 200 rpm. The operation pressure was reduced by 50% for each successive stage.

2.2.2. Chemical composition of REO and MD fractions

The chemical composition of REO and MD fractions was determined using a gas chromatography-mass spectrometry equipped with a flame ionization detector and a Carbowax capillary column (60 m × 0.25 mm, 0.25 μm). The oven temperature was held at 60 °C for 5 min and then increased 5 °C/min up to 240 °C. The carrier gas flow (He) was 1 mL/min. The injector and detector temperatures were 250 and 350 °C, respectively. The samples were diluted in *n*-hexane (1/100 μL) and 1 μL was injected. Identification of the compounds was performed by comparing the peak mass spectrum with the mass spectrum of pure standards. Relative concentrations were calculated according to peak area normalization using TurboMass 5.4.2 software.

2.2.3. Antioxidant activity – RSC

The method of Mezza et al. (2013) was used to evaluate the antioxidant activity of REO and MD fractions. Sample-hexane solutions (2 mL) prepared at 0.1 and 50 mg/mL were added to 2 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in hexane. After 120 min, the absorbance was measured at 517 nm. The blank was hexane and the control solution was prepared with 2 mL DPPH solution and 2 mL hexane. The RSC percentage was calculated as: ((AC–AS)/AC)*100, where AS is the absorbance of the sample solution containing antioxidant and AC is the absorbance of control solution.

IC_{50} was defined as the amount of sample (μL/mL) that produced a 50% decrease in the initial DPPH concentration. Lower IC_{50} values indicate higher free RSC.

2.2.4. Oxidative stability of SO

Samples of refined SO were supplemented with REO and MD fractions that exhibited a high free RSC. Sunflower oil without any additive (SO) was used as a control sample. SO supplemented with butylated hydroxytoluene (BHT) was prepared to compare its antioxidant activity against the natural antioxidants (REO and various fractions). REO and various MD fractions were added at 0.1 g/100 g while 0.02 g/100 g BHT was added, based on the maximum amount allowed in edible oils according to the Argentine Food Code (2012). Samples were placed in test tubes and stored uncovered in a dark place at 23 °C (room temperature). Samples of each product were removed from storage for chemical analyses at 0, 5, 15, 26, 46, 65, and 115 days.

The sample identifications were assigned as follows: SOREO, SO supplemented with REO; SOR1, SO supplemented with the stage R1 fraction; SOR4, SO supplemented with the stage R4 fraction; SOD4: SO supplemented with the stage D4 fraction; and SOBHT, SO enriched with BHT.

Peroxide value (PV) and conjugated dienes and trienes (CD and CT, respectively) were used as indicators to evaluate the oxidation of the stored samples. PV was analyzed according to the AOAC method and expressed as active oxygen milliequivalents (meqO_2/kg) (AOAC, 1980). CD and CT were measured in a UV–vis spectrophotometer (Biotraza

Table 1
Pressure for operating condition and distillates and residues percentages obtained by molecular distillation.

Stage N°	F[A]	Pressure (kPa)	g D/100 g F	g R/100 g F	g D/100 g REO	g R/100 g REO
1	REO	6.00	19.67 ± 0.30	80.33 ± 0.30	19.67 ± 0.30	80.33 ± 0.30
2	R1	3.00	20.24 ± 1.06	79.76 ± 1.06	16.27 ± 0.91	64.07 ± 0.62
3	R2	1.50	16.26 ± 0.42	83.74 ± 0.42	10.42 ± 0.37	53.65 ± 0.24
4	*R3*	0.75	14.89 ± 3.31	85.12 ± 3.32	7.99 ± 1.81	45.66 ± 1.57
Σ D/REO					54.35	

[A] Abbreviations. D = distillate, F = feeding, R = residue, REO = rosemary essential oil, R1 n = residue fraction of stage 1, R2 = residue fraction of stage 2, R3 = residue fraction of stage 3.

752, Instrumental Pasteur, Buenos Aires, Argentina) at 232 and 268 nm, respectively. The results are expressed as the extinction coefficient, E (1%, 1 cm) (COI, prueba espectrofotométrica en el ultravioleta. Document COI/T, & Madrid, 2001).

2.2.5. Statistical analyses

Analytical determination results are the average of three independent samples. The data were analyzed using Infostat software, version 2012.p (Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Córdoba, Argentina). Statistical differences were estimated by analysis of variance (ANOVA) at the 95% confidence level ($p < 0.05$). Less significant difference (LSD) test was used to detect pair-wise differences among the means. Principal component analysis (PCA) and biplot graphics were performed. Regression equations were used to determine the effect of the independent variable (time) on chemical oxidation indicators (PV). The regression analysis was performed by adjusting a simple linear model: $y = \beta_0 + \beta_1 . x$, where 'y' was the dependent variable (PV); and 'x' was the independent variable (time).

3. Results and discussion

3.1. Preparation and chemical characterization of MD fractions

Percentages of the distillate (g D/100 g F) and residue (g R/100 g F) ratios obtained, as a function of the amount of feed for each stage, are presented in Table 1. Percentages of the distillate (g D/100 g REO) and residue (g R/100 g REO), based on the amount of REO fed in the first stage, are also shown in Table 1. As a consequence of using four stages and low operating pressures, higher percentages of volatile compounds were obtained successfully in the distillate stream (54.35%) along with a residue (45.66%) containing fewer volatile compounds. Mezza et al. (2013) in a previous research reported lower distillate (18.44%) and residue (81.56%) streams, working under less severe operating conditions.

The chemical composition of REO and various molecularly distilled fractions are presented in Table 2. REO contained 35.23% monoterpene hydrocarbons where the major components were α-pinene, myrcene, camphene, and cymene; 56.53% oxygenated monoterpenes where the major constituents were 1,8-cineole and camphor; 2.60% sesquiterpene hydrocarbons (β-caryophyllene); 0.95% oxygenated sesquiterpenes (β-caryophyllene epoxide); and 1.72% diterpene hydrocarbons corresponding to (6E,8E,10E)-2,6,11,15-tetramethyl-2-6,8,10,14-β-hexadecapentaene. At the end of the MD process, 7.37% monoterpene hydrocarbons; 61.28% oxygenated monoterpenes; 10.82% sesquiterpene hydrocarbons; 3.68% oxygenated sesquiterpenes; and 7.00% diterpene hydrocarbons were found. The results demonstrate that MD can be used to produce fractions with variable compound concentrations.

The greatest concentration of α-terpineol obtained by Mezza et al. (2013) was 3.26 g/100 g found in the residue fraction from REO. The relatively higher pressures used in this research allowed increased α-terpineol concentrations, reaching 6.51, 9.22, and 11.47 g/100 g in R2, *R*3, and R4, respectively.

The volatile compound contents decreased progressively with successive stages because the most volatile compounds constituents evaporated in early stages. Consequently, D1 presented low concentrations of *cis*-sabinene hydrate, linalool, and camphor, but this distillated fraction had the most volatile compounds, like α-pinene, camphene, β-pinene, and myrcene.

Camphor and other less volatile compounds gradually increased in concentration in the distillate across the stages with a maximum in D4, because these less volatile compounds require more severe conditions to evaporate. The most severe conditions were used in the last stage and R4 had the highest concentrations of α-terpineol and *cis*-sabinene hydrate. It also had the highest concentrations of less volatile compounds like (6E,8E,10E)-2,6,11,15-tetramethyl-2–6,8,10,14-β-hexadecapentaene and caryophyllene epoxide. Both compounds were found only in REO and in the residue fractions (R1, R2, *R*3, and R4), which indicates that these both compounds can be separated by MD and concentrated in the residue fractions using the process condition presented in the current research.

3.2. Antioxidant activity of REO and REO fractions

IC_{50} values for REO, and distillate and residue fractions from all four MD stages, were significantly different (Table 3). D1 and D2 exhibited the lowest capacity to capture free radicals. In contrast, R4 displayed the highest capacity to capture free radicals, showing significant differences between the other samples. R2 and *R*3 had no statistically significant differences in IC_{50}.

Free-radical scavenging activity is mediated by an electron donor molecule (antioxidant). Phenols are H-donor molecules (Olmedo et al., 2013). Oregano, rosemary, and laurel essential oils with phenolic components have shown remarkable antioxidant activity because these compounds have a phenolic base (Olmedo, Asensio, & Grosso, 2015). Phenols with *ortho*-substitution of electron-donating alkyl or methoxy groups increase the stability of free radical, but the meta position has little or no effect on the antioxidant property of chemical structures. Fractions improved with oxygenated monoterpenes are more effective as antioxidant than phenolic fraction alone, suggesting a synergistic effect among these compounds (Asensio, Nepote, & Grosso, 2013). Therefore, oxygenated monoterpenes could have an important role in the antioxidant properties of composite fractions.

The association between the IC_{50} and chemical composition of fractions obtained by MD is presented in Fig. 1. The first principal component (PC1) accounted for 85.00% variation among samples while the second principal component (PC2) accounted for 13.80% variation. Both components, PC1 and PC2 exhibited 98.80% data variability.

The points with greater IC_{50} values were observed on the right side of Fig. 1 and associated with myrcene, limonene, 1,8-cineole, cymene, α-pinene, camphene, and β-pinene. These components were present at greater concentrations in D1, D2, and D3, which also had greater IC_{50} values; these samples also had poor antioxidant activity.

Points with lower IC_{50} values were observed on the left side of Fig. 1 and were associated with *cis*-sabinene hydrate, and α-terpineol. R3 and R4 samples exhibited higher concentrations of these compounds and lower IC_{50} values. MD process increased the content of *cis*-sabinene hydrate and α-terpineol (2.6 and 3.8 times, respectively) with respect to REO, particularly in R4. Both samples (R3 and R4) showed good antioxidant activity.

Bozin et al. (2007) reported a value of 3.82 μL mL^{-1} for REO, which is lower than observed in this study, but higher than IC_{50} values observed in the residues fractions (R1, R2, R3, and R4). Beretta et al. (2011) found that REO had the most activity during flowering, attributing this effect to the chemical composition and, specifically, the presence of *cis*-sabinene hydrate and α-terpineol.

3.3. Oxidative stability of SO with addition of REO fractions

The changes in PV, CD, and CT of the SO, SOREO, SOR1, SOR4, SOD4, and SOBHT samples during storage at 23 °C are shown in Fig. 2. In general, these variables increased with storage time in all samples. Significant differences in PV during storage were observed between samples from storage day 15. The SO samples exhibited the highest PV while SOBHT displayed the lowest PV.

D4, R1, and R4 fractions obtained by MD and REO had lower PVs than SO. All samples showed significant differences from 115 days of storage. PVs increased in the following order: SOBHT < SOR4 < SOR1 < SOREO < SOD4 < SO.

CD increased during storage from 3.16 to 13.18 in SO; 3.14 to 9.07 in SOREO; 3.16 to 9.83 in SOD4; 3.14 to 7.99 in SOR1; 3.16 to 7.00 in SOR4; and 3.14 to 5.90 in SOBHT. CD values increased in the following

Table 2
Chemical composition of the Rosemary Essential Oil and its fractions obtained by molecular distillation.

Compound[B]	Concentration (g/100 g)[A]								
	REO	D1	D2	D3	D4	R1	R2	R3	R4
Monoterpene hydrocarbons	**35.23**	**57.01***	**40.47***	**29.09**	**16.11**	**27.68**	**18.96**	**9.47**	**7.37**
α-pinene	15.77 ± 0.66**e**	28.68 ± 0.28**f**	18.55 ± 1.18**e**	9.96 ± 1.48 **cd**	5.04 ± 0.45**ab**	10.85 ± 2.65**d**	6.46 ± 1.00bc	3.17 ± 0.32**a**	2.60 ± 0.03**a**
Camphene	5.31 ± 0.25**e**	9.30 ± 0.09 g	6.50 ± 0.35**f**	3.70 ± 0.50 **cd**	1.96 ± 0.16**ab**	3.87 ± 0.85**d**	2.46 ± 0.41bc	1.24 ± 0.12**a**	1.03 ± 0.01**a**
β-pinene	1.53 ± 0.08**d**	2.52 ± 0.00**f**	1.99 ± 0.07**e**	1.22 ± 0.14 **cd**	0.67 ± 0.04**ab**	1.21 ± 0.23**c**	0.84 ± 0.17bc	0.41 ± 0.04**a**	0.34 ± 0.01**a**
Myrcene	5.65 ± 0.18**c**	8.05 ± 0.01**d**	7.74 ± 0.03**d**	5.68 ± 0.39**c**	2.92 ± 0.05**b**	4.91 ± 0.83**c**	3.44 ± 0.50 b	1.54 ± 0.10**a**	1.10 ± 0.07**a**
Limonene	2.90 ± 0.04**c**	3.65 ± 0.05**d**	4.01 ± 0.02**d**	3.34 ± 0.21 **cd**	2.07 ± 0.05**b**	2.76 ± 0.35**c**	2.26 ± 0.31 b	1.20 ± 0.09**a**	0.92 ± 0.04**a**
Cymene	4.07 ± 0.13**bc**	4.81 ± 0.03 **cd**	5.69 ± 0.02**d**	5.19 ± 0.35**d**	3.45 ± 0.09**b**	4.08 ± 0.52**bc**	3.50 ± 0.35 b	1.91 ± 0.17**a**	1.38 ± 0.04**a**
Oxygenated monoterpenes	**56.53**	**42.74**	**55.00**	**68.41***	**77.20***	**61.01***	**67.85***	**63.65***	**61.28***
1,8-cineole	29.24 ± 0.31**c**	32.97 ± 0.18**d**	37.29 ± 0.52**d**	34.24 ± 1.30**d**	22.77 ± 0.46**b**	27.07 ± 2.35**c**	22.39 ± 1.64**b**	13.75 ± 1.09**a**	11.33 ± 0.16**a**
Camphor	20.33 ± 0.24**c**	8.81 ± 0.14**a**	15.71 ± 0.60**b**	27.99 ± 1.97**de**	40.35 ± 0.44 g	24.88 ± 0.47**d**	31.91 ± 1.01**f**	31.88 ± 2.28**f**	29.56 ± 1.09**ef**
Linalool	2.56 ± 0.10**b**	0.50 ± 0.00**a**	1.02 ± 0.16**a**	3.17 ± 0.70**c**	6.34 ± 0.05**f**	3.32 ± 0.31**c**	4.58 ± 0.11**d**	5.52 ± 0.31**e**	5.40 ± 0.20**e**
cis-Sabinene hydrate	1.35 ± 0.09**b**	0.20 ± 0.00**a**	0.42 ± 0.05**a**	1.21 ± 0.26**b**	2.76 ± 0.02**c**	1.70 ± 0.29**b**	2.46 ± 0.03**c**	3.28 ± 0.16**d**	3.52 ± 0.21**d**
α-terpineol	3.05 ± 0.15**bc**	0.26 ± 0.01**a**	0.56 ± 0.05**a**	1.80 ± 0.40**ab**	4.98 ± 0.11**de**	4.04 ± 0.50 **cd**	6.51 ± 0.07**e**	9.22 ± 0.5**f**	11.47 ± 1.21 g
Sesquiterpene hydrocarbons	**2.60**	**0.11**	**0.24**	**0.90**	**2.71***	**3.46***	**5.03***	**8.68***	**10.82***
β-caryophyllene	2.60 ± 0.02**bc**	0.11 ± 0.00**a**	0.24 ± 0.06**a**	0.90 ± 0.31**ab**	2.71 ± 0.02**bc**	3.46 ± 1.11 **cd**	5.03 ± 0.95**d**	8.68 ± 0.44**e**	10.82 ± 0.41**f**
Oxygenated sesquiterpenes	**0.95**	**ND**	**ND**	**ND**	**ND**	**1.17***	**1.24***	**2.95***	**3.68***
β-caryophyllene epoxide	0.95 ± 0.18[a]	ND	ND	ND	ND	1.17 ± 0.82**a**	1.24 ± 0.45**a**	2.95 ± 0.92**b**	3.68 ± 0.57**b**
Diterpene hydrocarbons	**1.72**	**0.00**	**0.00**	**0.00**	**0.00**	**2.27***	**2.38***	**6.08***	**7.00***
(6E,8E,10E)-2,6,11,15-Tetramethyl-2,6,8,10,14-hexadecapentaene	1.72 ± 0.33[a]	ND	ND	ND	ND	2.27 ± 1.58**a**	2.38 ± 1.23**a**	6.08 ± 1.85**b**	7.00 ± 1.34**b**

[A] Abbreviation. REO = rosemary essential oil, D1 = distillate fraction of stage 1, D2 = distillate fraction of stage 2, D3 = distillate fraction of stage 3, D4 = distillate fraction of stage 4, R1 = residue fraction of stage 1, R2 = residue fraction of stage 2, R3 = residue fraction of stage 3, R4 = residue fraction of stage 4. ND = not detected.

[B] Different letters in the same row denote significant difference, LSD Fisher Test (α = 0.05).

* Denotes fractions enriched in these components with respect to REO.

Table 3
IC_{50} values of rosemary essential oil and its fractions obtained by molecular distillation.

Sample[A]	IC_{50} (μL mL^{-1})[B]	
REO	4.39 ± 0.07	d
D1	> 100	g
D2	> 100	g
D3	29.17 ± 0.22	f
D4	12.81 ± 0.11	e
R1	3.20 ± 0.09	c
R2	2.35 ± 0.04	b
R3	2.28 ± 0.08	b
R4	1.82 ± 0.10	a

[A] Abbreviations. REO = rosemary essential oil, D1 = distillate fraction of stage 1, D2 = distillate fraction of stage 2, D3 = distillate fraction of stage 3, D4 = distillate fraction of stage 4, R1 = residue fraction of stage 1, R2 = residue fraction of stage 2, R3 = residue fraction of stage 3, R4 = residue fraction of stage 4.
[B] Different letters denote significant difference between samples, LSD Fisher Test (α = 0.05).

order: SOBHT < SOR4 < SOR1 < SOREO < SOD4 < SO.

All samples showed a significant difference (α = 0.05) in CD during storage. CD values in SOBHT were constant until day 65. Residue samples had constant CD values until day 26. SO and SOBHT samples had the highest and lowest CD values during storage, respectively.

CT increased during storage from 1.85 to 4.99 in SO; 1.86 to 3.62 in SOREO; 1.85 to 3.29 in SOD4; 1.86 to 2.91 in SOR1; 1.86 to 2.24 in SOR4; and 1.85 to 2.05 in SOBHT. SOR4 and SOBHT showed no significant change during 65 days of storage. SO exhibited the highest CT during storage. Furthermore, SO and SOD4 showed the greatest increment rate for CD and CT. The residue fractions displayed lower PV, CD, and CT than REO and the distillate fractions. Storage stability results suggest the residue fractions have important antioxidant activity.

Quiroga, Riveros, Zygadlo, Nepote, and Grosso (2011) reported a negative association between the oxidation indicators in canola oil (PV and AV) and cis and trans-sabinene hydrate, terpinen-4-ol, α-terpinene, 1,8-cineole, β-trans-ocimene, sabinene, and γ-terpinene in essential oil from oregano species, which indicated these compounds have antioxidant properties. Olmedo et al. (2014) reported that the residue samples separated by two-stage MD (R1 and R2) exhibited higher lipid oxidation values during storage than distillate fractions (D1 and D2). Therefore, the distillate fractions were more effective antioxidants than the residues. Those authors also reported that γ-terpinene, α-terpinene, sabinene, and β-phellandrene made up 67.45 and 55.3% of D1 and D2, respectively. In the present study, oxygenated monoterpenes, like cis sabinene hydrate and α-terpineol, in residue fraction, especially in R4, are, probably, the molecules responsible for the antioxidant activity of REO.

The regression equation for PV (SO, SOREO, SOR1, SOR4, SOD4, and SOBHT) during storage at 23 °C is presented in Table 4. The PVs exhibited positive slopes, indicating a linear increase with storage time. All regression coefficients (R^2) were higher than 0.98 indicating that the regression models were a good predictor of PV. Therefore, these regression equations could be used to predict the effect of storage time at 23 °C for SOs. PV variable showed significant differences in regression slopes among samples during storage. According to the regression equations, SO and SOD4 displayed greater degrees of deterioration, based on a steeper slope for PV. The distillate fractions exhibited fewer antioxidant compounds than the residue fractions. SOBHT showed the shallowest slope for all the dependent variables (PV, CD, and CT), as shown in Table 4.

According to the Argentine Food Code (2012), 10 meqO_2/kg is the maximum level of PV allowed for edible oils. This value might be a useful quality endpoint for SO supplemented with antioxidant-containing fractions. Therefore, the SO shelf-life was estimated to end at the time PV reached 10 meqO_2/kg using the PV-time linear regressions. A PV greater than 10 meqO_2/kg was reached after 26 days for SO, 30 days for SOD4, 36 days for SOREO, 38 days for SOR1, 44 days for SOR4, and 52 days for SOBHT. Therefore, SOREO, SOD4, SOR1, SOR4 and SOBHT displayed a longer shelf-life than SO. The 10 meqO_2/kg of PV recommended by the Argentine Food Code (2012) was reached within a few days because the samples were exposed to the air during storage. However, the results are valid for a comparative analysis and indicate residue fractions supplemented with natural antioxidants provide protection against lipid oxidation in SO.

3.4. Relation between compounds in REO and their antioxidant capacity

α-Terpineol and *cis*-sabinene hydrate are oxygenated monoterpenes (Beretta et al., 2011). Both of them were the compounds responsible for antioxidant activity in the extracts in accordance with Quiroga, Asensio, & Nepote (2014).

In the residue fractions, MD achieved three and fourfold increases in *cis*-sabinene hydrate and α-terpineol, respectively. The concentrations of *cis*-sabinene hydrate and α-terpineol were 3.52 and 11.47 g/100 g in

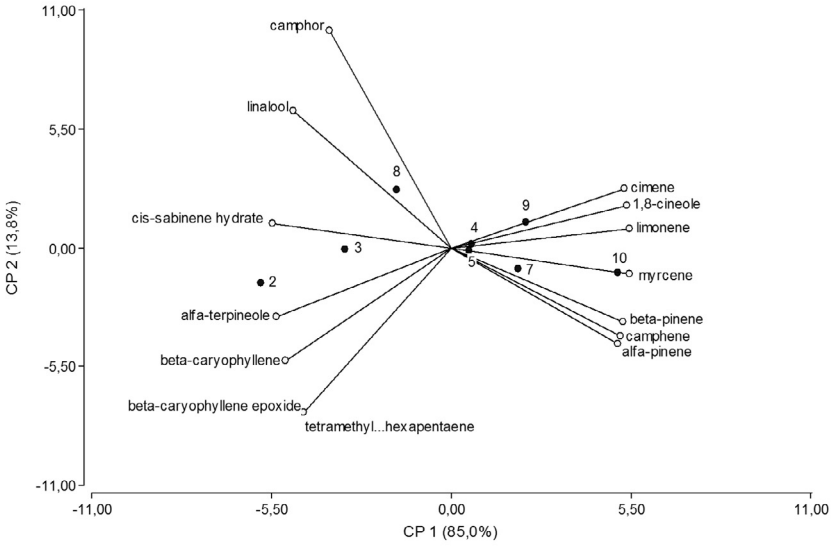

Fig. 1. Biplot of first and second principal components from PCA of rosemary essential oil components in association with the IC_{50} values. Compounds represented by vectors and IC_{50} levels represented by points. Compounds: α-pinene; camphene; β-pinene; myrcene; limonene; cymene; 1,8-cineole; camphor; linalool; *cis*-sabinene hydrate; α-terpineol; β-caryophyllene; β-caryophyllene epoxide; (6E,8E,10E)-2,6,11,15-Tetramethyl-2,6,8,10,14-hexadecapentaene. IC_{50} levels: 1: (0.00–1.00 μL/mL), 2: (1.01–2.00 μL/mL), 3: (2.01–2.50 μL/mL l), 4: (2.51–3.00 μL/mL), 5: (3.01–3.50 μL/mL), 6: (3.51–4.00 μL/mL), 7: (4.01–5.00 μL/mL), 8: (5.01–20.00 μL/mL), 9: (20.01–40.00 μL/mL), 10: (> 40.01 μL/mL).

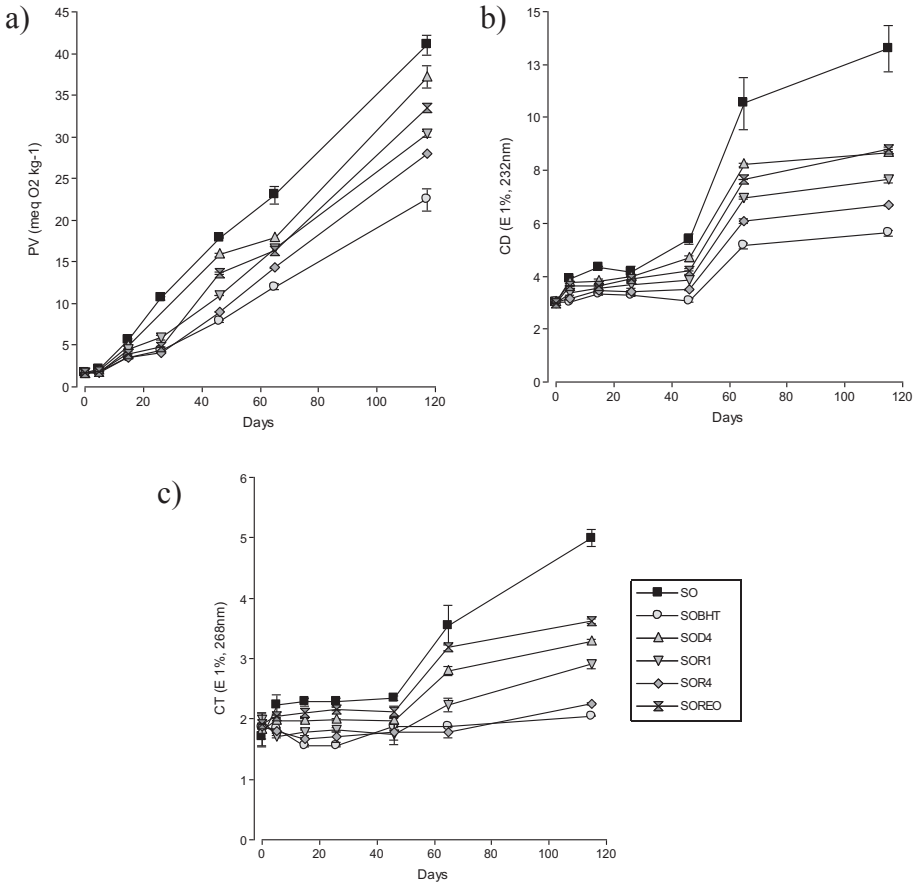

Fig. 2. a) Peroxide values (PV), b) Conjugated dienes (CD), and c) Conjugated trienes (CT) measured in sunflower oil during storage time at 23 °C.

Table 4
Regression equation and R^2 estimated using the results of peroxide value (PV), conjugated dienes (CD), and conjugated trienes (CT) during storage time.

Dependent variable	Samples[B]	Linear regression coefficients[A]			
		β_0	β_1[C]		R^2
PV	SOBHT	0.53695	0.18259	a	0.98680
	SOR4	-0.12733	0.23249	b	0.98446
	SOR1	0.46433	0.24963	bc	0.99335
	SOREO	-0.01202	0.27821	cd	0.98242
	SOD4	0.72973	0.30563	d	0.98940
	SO	1.18300	0.34150	e	0.99680
CD	SOBHT	2.91034	0.02642	a	0.80610
	SOR4	2.87534	0.03664	ab	0.83103
	SOR1	2.93000	0.04541	bc	0.86158
	SOREO	2.99168	0.05463	c	0.86397
	SOD4	3.08847	0.06194	c	0.88565
	SO	2.94538	0.09165	d	0.90079
CT	SOBHT	1.67600	0.00302	a	0.45705
	SOR4	1.71061	0.00300	a	0.26907
	SOR1	1.62800	0.00902	b	0.66879
	SOREO	1.76254	0.01215	bc	0.78285
	SOD4	1.73483	0.01658	c	0.82476
	SO	1.71622	0.02637	d	0.88338

[A] Coefficients for the regression equation: y = βo + β1x, where y = dependent variable (PV, CD, or CT) and x = independent variable (storage days).

[B] Samples. Sunflower oil (SO), sunflower oil enriched with rosemary essential oil (SOREO), sunflower oil enriched with residue fraction of stage 1 (SOR1), sunflower oil enriched with residue fraction of stage 4 (SOR4), sunflower oil enriched with distillate fraction of stage 4 (SOD4), and sunflower oil enriched with commercial antioxidant BHT (SOBHT).

[C] Different letters denote significant difference in regression slopes between samples (α = 0.05).

R4, respectively, compared with 1.35 and 3.05 g/100 g in REO. Bozin et al. (2007) reported that the compounds responsible for neutralization of DPPH radicals were oxygenated monoterpenes and sesquiterpenes from REO and sage essential oil. The antioxidant properties of oxygenated monoterpenes, like α-terpineol and sabinene hydrates, have been detected previously using different methods (Bicas, Neri-Numa, Ruiz, & De Carvalho, 2011; Quiroga, Grosso, & Nepote, 2013). Kulisic, Radonic, Katalinic, and Milos (2004) reported that the oxygen-containing fraction was more effective as an antioxidant than phenolic fractions. It is accepted that the mechanism of action is through hydroxyl (OH·) radical (Forester & Wells, 2011). However, specific oxidation reaction mechanism for these monoterpenes is still unknown.

4. Conclusions

Results from the present study indicate the addition of REO fractions to SO improved the stability of this food product, delaying lipid oxidation. Residue fractions prepared by MD have lower IC_{50} for REO and better antioxidant activity in comparison with REO and distillate fractions. The residue fractions could be used as natural antioxidants for SO and other vegetable oils, increasing their shelf-life, improving their stability, and preventing loss of their sensory and nutritional qualities.

Antioxidant compounds in the analyzed extracts that were identified as oxygenated monoterpenes, specifically α-terpineol and *cis*-sabinene hydrate, could be responsible for the antioxidant activity of REO and residue fractions.

This research renews interest in using natural antioxidants by the food industry. Natural antioxidants could be used to replace synthetic antioxidants in high-lipid food products.

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