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Supercritical carbon dioxide extraction of antioxidants from rosemary (*Rosmarinus officinalis*) leaves for use in edible vegetable oils

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Abstract: Supercritical extraction was employed to produce rosemary (*Rosmarinus officinalis L.*) extracts with different composition and antioxidant activity. CO_2 was utilized as supercritical solvent and diverse extraction conditions (temperature, pressure, amount of cosolvent and fractionation scheme) were applied. The extracts with higher antioxidant content were selected to study their capability as natural antioxidant of several commercial edible vegetable oils. Linseed oil (LO), grape seed oil (GO) and sesame oil (SO) were oxidized under Rancimat conditions in presence of 0, 100, 200 and 300 mg/kg of selected extracts. Antioxidant activity index (AAI) was estimated as the ratio of induction time in presence of extracts to induction time in absence of extract. Induction time in absence of extracts was 3.3, 7.9 and 23.4 h for LO, GO and SO, respectively. Regardless of these different susceptibilities, the highest AAI for the three oils was obtained for the extract with the highest antioxidant-enrichment (33.25% carnosic acid plus carnosol) and added at the highest level (300 mg/kg). However, at such conditions, the AAI was significantly higher ($p<0.001$) for LO (3.5), followed by SO (2.2) and the poorest value was for GO (1.1). Therefore, the magnitude of the AAI depended on the antioxidant-enrichment of the extracts, the level of addition within the oils, but also on the own individual oils.

Key words: Rosemary, Antioxidant, Supercritical extraction, Vegetable oil

1 INTRODUCTION

Antioxidant compounds in food play a very important role. Oxidation is one of the major causes of chemical spoilage, resulting in rancidity and/or deterioration of the nutritional quality, color, flavor, texture and safety of foods. Modern consumers ask for natural products, free of synthetic additives, and therefore several spices and some herbs have received increased attention as sources of effective natural antioxidants[1, 2].

Rosemary (*Rosmarinus officinalis L.*) has been recognized as one of the *Lamiaceae* plants with large antioxidant activity. Main substances associated with the antioxidant activity are the phenolic diterpenes such as carnosol, rosmanol, carnosic acid, methyl carnosate, and phenolic acids such as the rosmarinic and caffeic acids[3–6]. Particularly, carnosic acid and carnosol are the most abundant antioxidant compounds present in rosemary[7].

Indeed, supercritical fluid technology is the best innovative method to recover bioactive compounds for use as supplements for functional foods[8, 9]. Different authors[10, 11] compared rosemary supercritical extracts with those obtained using liquid solvents (ethanol and hexane) or hydrodistillation, and demonstrated the superior antioxidant activity of the supercritical extracts.

Recently, the European Commission published Directive 2010/67/EU of 20 October 2010[12] and informed on the safety of rosemary extracts when used as an antioxidant in foodstuffs. Such document establishes appropriate specifications to authorize rosemary extracts as a new food additive for use in foodstuffs, and assigned E 392 as its E number. Moreover, several types of production process are described, using solvent extraction (ethanol, acetone and hexane) and also supercritical CO_2 extraction. Thus, according to Directive 2010/67/EU, supercritical rosemary extracts for use as food additive should contain more than 13% w/w of antioxidant compounds (carnosic acid + carno-

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sol) and the antioxidant/volatiles ratio should be greater than 15.

There are several works reporting the antioxidant activity of rosemary extracts obtained by conventional methods (hydro-distillation and liquid solvent extraction) in different meat products[13−17] and diverse vegetable oils, such as sunflower oil[18], soybean oil[19, 20], peanut oil[21] or *Camelina sativa* oil[22]. Nevertheless, studies specifically focused on the use of supercritical extracts are scarce. As recent example, Bavovic et al.[23] studied the antioxidant activity of supercritical fluid extracts of rosemary, sage, thyme and hyssop extracts in preventing oxidation of sunflower oil. By means of the measurement of the oil peroxide value, the authors concluded that the best antioxidant effect was obtained with rosemary and sage. Supercritical rosemary extracts have been also shown as efficient antioxidant in wheat germ oil, leading to better results than those extracts obtained from Soxhlet procedure[24]. Recently, Martin et al.[25] tested the use of supercritical rosemary extract in the protection of n-3 concentrates from fish oils, showing successful results in combination with α-tocopherol.

In this work, rosemary supercritical extracts with different concentration of antioxidant compounds were produced, by using diverse extraction conditions such as temperature, pressure, amount of co-solvent (ethanol) and fractionation scheme. The antioxidant power of the extracts was evaluated by the DPPH test and also the extracts were assessed in terms of the specifications stated in Directive 2010/67/EU.Furthermore, the antioxidant power of supercritical rosemary extracts was evaluated by studying under Rancimat conditions their use as potential antioxidants of three different vegetable oils: linseed oil (LO), grapeseed oil (GO) and sesame oil (SO). The different level of polyunsaturated fatty acids (PUFA) of these oils, their nature as n-3 or n-6 fatty acids and the own presence of natural antioxidant compounds, might determine different oxidative susceptibilities of these oils, as well as different antioxidant power of exogenous-added compounds to control their loss of value. Previous data on the use of rosemary extracts on the stabilization of GO, SO or LO have not been found in the literature.

2 EXPERIMENTAL PROCEDURES

2.1 Chemicals and samples

2, 2- diphenyl-1-picryl hydrazyl hydrate (DPPH, 95% purity), camphor (>97%), bornyl acetate (95%) and linalool (>97%) were purchased from Sigma-Aldrich. Carnosic acid (≥ 96%) and carnosol was purchased from Alexis Biochemical. 1,8 cineole (98%) and borneol (>99%) were purchased from Fluka. Ethanol and phosphoric acid (85%) were HPLC grade from Panreac. Acetonitrile was HPLC grade from Lab Scan (Dublin, Ireland). CO_2 (N38) was supplied from Air Liquid.

LO, GO and SO were purchased from a local market. Fatty acid compositions for these oils (according to specifications of the producer) are given in Table 1. The rosemary (*Rosmarinus officinalis L.*) raw material consisted of dried leaves (water content<5% w/w) obtained from an herbalist's producer (Murcia, Spain). The sample was ground in a cooled mill. Sample particle size was in the range of 200 and 600 μm.

Table 1 Fatty acid composition and oxidative stability of vegetable oils.

	LO	GO	SO
Fatty acid composition[a]			
SFA (%)[b]	9	12	14
MUFA (%)[c]	19	19	40
PUFA (%)[d]	72	69	46
n-6 linoleic acid	19	68	44
n-3 linolenic acid	53	1	2
PUFA/SAT	8	5.8	3.3
Oxidative stability			
IT (h) [e,f]	3.25 ± 0.05^{z}	7.9 ± 0.52^{y}	23.35 ± 0.24^{x}

[a] According to specifications of the producer

[b, c, d] SFA (saturated fatty acids), MUFA (monounsaturated fatty acids) and PUFA (polyunsaturated fatty acids)

[e] Without addition of supercritical rosemary extracts.

[f] Different letters between values means that averages were significantly different

2.2 Supercritical extraction

Extractions were carried out using a supercritical fluid pilot-plant (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction cell and two different separators (S1 and S2), each of 0.5 L capacity with independent control of temperature (±2℃) and pressure (±0.1 MPa). The extraction equipment also includes a recirculation system, where CO_2 is condensed, pumped up to the desired extraction pressure and heated up to the selected extraction temperature.

The temperature of the extraction cell and separators was maintained at 40℃ and CO_2 flow rate was 60 g/min in all experimental assays. In selected assays, fractionation of the extracted material was accomplished by setting the pressure of the first separator (S1) to 10 MPa, while the second separator (S2) was maintained at the recirculation system pressure (5 MPa). In this case, two different samples were collected: one sample from S1 cell and the other from S2 cell. When no fractionation of the extract was accomplished, S1 was set to the recirculation system pressure and thus, only one sample was recovered from S1. Extraction conditions were selected on the basis of previous studies reported in the literature[10, 26-32] with respect to the supercritical fluid extraction (SFE) of rosemary leaves to produce antioxidant fractions, and are explained in detail as follows.

Extraction 1: Extractor pressure was 30 MPa, extraction time 360 min, and no fractionation of the extracted material was accomplished. Only one sample was collected from S1 separator (M1 sample).

Extraction 2: Extractor pressure was 30 MPa and fractionation of the extracted material was accomplished during the first 60 min. Then, extraction continued for 300 min without fractionation. Two samples were collected: one from S1 (M2-1 sample) and the other from S2 (M2-2).

Extraction 3: Extractor pressure was 15 MPa and 5% w/w ethanol was employed as cosolvent. No fractionation of the extracted material was accomplished during 180 min of extraction. Only one sample was collected from S1 separator (M3 sample).

Extraction 4: Extractor pressure was 15 MPa and 10% w/w ethanol was employed as cosolvent. No fractionation of the extracted material was accomplished during 180 min of extraction. Only one sample was collected from S1 separator (M4 sample).

Extraction 5: Extractor pressure was 15 MPa and no fractionation was carried out. First (60 min) no cosolvent was employed and then (120 min) 10% w/w ethanol was used Two samples were collected from S1 separator, corresponding to the first (M5-1 sample) and second (M5-2 sample) extraction periods.

Extraction 6: The residual plant matrix from Extraction 1 was utilized as raw material in this experiment. Extractor pressure was 15 MPa and 10% w/w ethanol as cosolvent was employed. Extraction time was 180 min. No fractionation was accomplished and thus, one sample was collected in S1 separator (M6 sample).

2.3 GC-MS analysis

The essential oil compounds of samples were determined by GC-MS-FID using 7890A System (Agilent Technologies, U.S.A.), comprising a split/splitless injector, electronic pressure control, G4513A auto injector, a 5975C triple-Axis mass spectrometer detector, and GC-MS Solution software. The column used was an Agilent 19091S-433 capillary column, 30 m × 0.25 mm I.D. and 0.25 μm phase thickness. Helium, 99.996% was used as a carrier gas at a flow of 29.4 ml/min and inlet pressure of 200 MPa. Oven temperature programming was 60℃ isothermal for 4 min then increased to 106℃ at 2.5℃/min and from 106℃ to 130℃ at 1℃/min and finally from 130℃ to 250℃ at 20℃/min, this temperature was kept constant for 10 min. Sample injections (1 μl) were performed in split mode (1:10). Injector temperature was 250℃ and MS ion source and interface temperatures were 230 and 280℃, respectively. The mass spectrometer was used in TIC mode, and samples were scanned from 40 to 500 amu. Key volatiles were identified by comparison with standard mass spectra, obtained in the same conditions and compared with the mass spectra from library Wiley 229. The rest of compounds were identified by comparison with mass spectra from Wiley 229 library. A calibration curve was employed to quantify each of the key volatiles. GC-MS analyses were carried out by duplicate and the average standard deviation obtained was ± 0.08%.

2.4 HPLC analysis

Carnosic acid and carnosol content in the samples were determined using an HPLC (Varian Pro-star) equipped with a Microsorb-100 C_{18} column (Varian) of 25 cm × 4.6 mm and 5 μm particle size. The analysis is based on the work of Almela et al.[33]. The mobile phase consisted of acetonitrile (solvent A) and 0.1% of phosphoric acid in water (solvent B) applying the following gradient: from 0 min to 8 min, 23% A; increasing from 8 min to 25 min up to 75% A; kept constant during 15 min, and from 40 min to 45 min, initial conditions were gained (23% A). The flow rate was constant at 0.7 ml/min. Injection volume was 20 μl and the detection was accomplished by using a diode array detection system (Varian) storing the signal at a wavelength of 230, 280 and 350 nm. Samples were analyzed by HPLC in duplicate and the obtained average standard deviation was ± 0.13%.

2.5 Antioxidant activity by the DPPH test

The method consists in the neutralization of free radicals of DPPH by an antioxidant sample[34]. An aliquot (50 μl) of ethanol solution containing 5-30 μg/ml of rosemary extract, was added to 1.950 μl of DPPH in ethanol (23.5 μg/ml) pre-

pared daily. Reaction was completed after 3 h at room temperature and absorbance was measured at 517 nm in a Nanovette Du 730 UV spectrophotometer (Beckman Coulter, USA). The DPPH concentration in the reaction medium was calculated from a calibration curve determined by linear regression ($y = 0.0265 \cdot x$; $R^2 = 0.9998$; 6 calibration points from 0 to 40 μg/mL). Ethanol was used to adjust zero and DPPH-ethanol solution as a reference sample. The amount of extract necessary to decrease the initial DPPH concentration by 50% or EC_{50} (μg/ml) was determined and employed to value the antioxidant power of the sample; the lower the EC_{50}, the higher the antioxidant power.

2.6 Antioxidant activity under Rancimat test

Selected rosemary extracts were added to vegetable oils at 0, 100, 200 and 300 mg/kg. Oils (3 g) were subjected to accelerated oxidative conditions by a Metrohm Rancimat model 743 (Herisau, Switzerland) at an airflow rate of 20 L/h and at 100℃. The conductivity measuring cells contained 60 mL of distilled water. The induction time (IT) was automatically determined as the inflection point of the generated plot of conductivity (μS/cm) of the water versus time (h). Analyses were performed in duplicate. The antioxidant activity index (AAI) was estimated as:

AAI = IT with antioxidant/IT without antioxidant

2.7 Statistical analysis

Experimental supercritical extractions were carried out by duplicate in the SFE system. Standard deviations of extraction yields obtained were calculated as follows:

$$StD = \sqrt{\frac{1}{2} \times \left[(x_1 - \bar{x})^2 + (x_2 - \bar{x})^2 \right]} \quad (1)$$

Being x_1 and x_2 the values obtained in each of the experiments and $\bar{x}$ the corresponding average value.

Quantification of carnosic acid and key volatile oil compounds together with the antioxidant activity tests were also carried out by duplicate, employing the mixture of extracts obtained in the duplicate extraction assays. Eq. (1) was applied in order to test the reproducibility of the data obtained.

The effect of the factors "extract", "concentration of extract", and "type of oil" on the AAI value was evaluated by one-way analysis of variance by means of the SPSS 17.0 statistical package (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p \leq 0.05$. When the effect of any of the factors was significant, differences between groups were analyzed by Tukey's posthoc test.

3 RESULTS AND DISCUSSION

3.1 Supercritical rosemary extracts

The different conditions applied in the supercritical rosemary extraction were target to produce a sample with high content of antioxidant substances and low content of volatile oil compounds, but also with favorable production conditions such as high extraction yield, no cosolvent or low amounts of cosolvent consumption, and short extraction time. Table 2 shows the extraction yield, the carnosic acid content and the total content (% w/w) of key volatiles (borneol, bornyl acetate, camphor, 1,8-cineol and verbenone) of the supercritical rosemary extracts produced in the Extractions 1 to 6 defined before. Moreover, the normalized composition (% peak area) of the key volatile oil compounds is detailed in Table 3. Further, low amounts of carnosol (<3% w/w) were obtained in all samples.

As can be observed from Table 2, higher carnosic acid

Table 2 Extraction yield, carnosic acid and key volatiles content (% w/w) in the supercritical rosemary samples produced.

Ext.	Sample	Yield (g extract / g rosemary leaves × 100)[a]	Carnosic acid content (% w/w)[b]	Key volatiles content (% w/w)[c]
1	M1	4.52 ± 0.17	10.89	12.79
2	M2-1	2.83 ± 0.18	16.90	13.59
2	M2-2	1.53 ± 0.22	3.12	21.70
3	M3	7.26 ± 0.27	25.66	10.42
4	M4	13.44 ± 0.32	14.18	4.69
5	M5-1	1.42 ± 0.30	2.00	36.92
5	M5-2	3.02 ± 0.19	28.49	4.81
6	M6	4.93 ± 0.25	30.69	2.04

[a] mean standard deviation < 0.24

[b] values reported correspond to average value between duplicates; mean standard deviation < 0.53

[c] values reported correspond to average value between duplicates; mean standard deviation < 0.41

Table 3 Normalized (% peak area) composition of key volatile compounds identified in rosemary supercritical extracts[a].

Ext	Sample	1,8 cineole	Camphor	Borneol	Verbenone	Bornyl acetate
1	M1	66.75	22.83	8.45	n.d.[b]	1.97
2	M2-1	64.43	23.96	5.78	4.14	1.69
3	M2-2	48.28	32.29	10.44	7.27	1.71
3	M3	54.82	28.12	8.62	6.20	2.25
4	M4	56.23	27.95	9.44	6.38	n.d.
5	M5-1	58.40	19.62	6.75	9.20	1.15
5	M5-2	59.98	24.56	9.54	5.92	n.d.
6	M6	61.23	24.01	14.76	n.d.	n.d.

[a] deviations between two injections < 0.08%
[b] n.d. = not detected

contents seemed to be obtained when ethanol was employed as CO_2 cosolvent (M3, M5-2 and M6 samples). Furthermore, considering the concentration (% w/w) of the key essential oil components, samples obtained with ethanol as CO_2 cosolvent also seemed to be those with lower essential oil content (M4, M5-2 and M6 samples). In the case of samples M5-2 and M6, the low content of essential oil compounds could be attributed to the fact that, in both experiments, the plant matrix was previously extracted with pure CO_2 and thus, essential oil substances were almost exhausted. On the other side, the high yield obtained in Extraction 4 (13.44%) supposes a high co-extraction of other substances and thus, the concentration of both carnosic acid and volatile oil compounds was considerably reduced.

As expected, due to the fractionation procedure accomplished in Extraction 2, the extract collected in S1 (M2-1) seemed to contain higher amounts of carnosic acid and lower amounts of volatile oil compounds than the sample collected in S2 (M2-2). Further, in comparison with the extract obtained without fractionation (Extraction 1), M2-1 sample seemed to contain higher amounts of carnosic acid than M1 and both samples contained similar amounts of volatile oil components. Nevertheless, extraction yield seemed to be lower in the case of the M2-1, i.e. the sample with higher carnosic acid content.

Based on the SFE assays carried out in this work, higher amounts of antioxidants (e.g. carnosic acid) might be obtained only when a polar co-solvent (ethanol) is employed in the supercritical CO_2 extraction procedure. At this respect, if no ethanol is utilized, fractionation of the extract might improve the antioxidant activity of one of the fractions collected, but process yield might be noticeably reduced.

The rosemary supercritical samples selected to carry out the oxidative stability test were M1, M2-1 and M6. M1 and M2-1 were selected since both samples were produced without using ethanol as cosolvent. This is an important factor to be considered to evaluate a commercial rosemary supercritical extract production, since evaporation of cosolvent is an expensive task to be accomplished. As mentioned before, M2-1 seemed to contain larger amounts of carnosic acid but extraction yield was reduced from 4.42% to 2.83%.

From samples produced using ethanol as CO_2 cosolvent, M6 was selected since it was the sample that seemed to contain the highest carnosic acid content and lower key volatiles content. Furthermore, it should be considered that this sample was produced by the extraction of the same plant matrix utilized in Extraction 1. That is, Extraction 6 (15 MPa, 40℃, and 10% cosolvent) was accomplished after Extraction 1 (30 MPa, 40℃, and no cosolvent) and two extracts were obtained from the same amount of plant matrix processed, one with 4.52% yield and 10.89% w/w carnosic acid, and the other with 4.93% yield and 30.69% w/w carnosic acid.

Table 4 shows the EC_{50} value determined for samples M1, M2-1 and M6, using the DPPH test. As expected, the EC_{50} value tended to decrease as carnosic acid content increased. That is, the antioxidant power of the samples tended to increase with the increasing content of the main antioxidant substance (carnosic acid) present in the extracts. Also given in this table are the carnosic acid + carnosol content (% w/w) and the ratio antioxidant/key volatiles.As can be observed from the table, M6 extract satisfied Directive 2010/67/EU to authorize it as a food additive: carnosic acid + carnosol content greater than 13% w/w and the ratio antioxidants/key volatile compounds greater than 15. With respect to samples M1 and M2-1, it is clear that the main problem is related with the high content of volatile oil compounds. That is, a deodorization process should be accomplished to these samples in order to reduce the key volatile oil content and then satisfy Directive 2010/67/EU requirements. Indeed, the low content

Table 4 EC_{50} values, carnosic acid + carnosol content (% w/w) and antioxidants/key volatiles ratio obtained for supercritical rosemary samples M1, M2-1 and M6.

Rosemary extract	EC_{50} value[a] (μg/ml)	carnosic acid + carnosol (% w/w)	antioxidants/ key volátiles ratio
M1	32.97	11.94	0.93
M2-1	15.91	18.80	1.38
M6	9.8	33.25	16.29

[a] values reported correspond to average value between duplicates; mean standard deviation < 1.1

of volatile oil attained in M6 is a consequence of the previous SFE step (Extraction 1) carried out over the same plant matrix.

3.2 Antioxidant activity by Rancimat test

The antioxidant activity index (AAI) of selected rosemary supercritical extracts M1, M2-1 and M6 added to LO, GO and SO is shown in Fig. 1 as a function of the amount of extract added to the oil (mg rosemary extract/kg oil). In general, the higher the level of addition of rosemary extract the higher the AAI, regardless of the specific extract and the vegetable oil oxidized [p (concentration) = 0.026)]. Nevertheless, the magnitude of such AAI was different depending on the specific rosemary extract [p (extract) = 0.019]. A general trend was evidenced as increasing antioxidant activity in the following order: M1 < M2-1 < M6. In order to find a relationship of this result with the composition of the extracts, the AAI values for each individual extract at all assayed concentrations (100, 200 and 300 mg/kg) and for the three oils were considered all together and an average AAI was estimated. Such value was plotted against the specific level of carnosic acid plus carnosol content of each individual extract (Fig. 2). As it can be observed, a strong linear correlation was found ($r^2 = 0.9999$), which suggested that the antioxidant power of each extract in vegetable oils was determined by the specific content of carnosic acid plus carnosol content of the extracts, regardless of the level of addition of the extracts within the oils and regardless of the specific vegetable oil assayed. Therefore, the process of production of the M6 extract by the applied supercritical fluid conditions, which gave the rosemary extract with the highest concentration of antioxidant compounds, was reflected in the highest prolongation of the induction time of lipid oxidation of the assayed vegetable oils. At this respect, it should be noted that the use of the M6 extract would allow reaching the same antioxidant effect, but at lower concentrations, than the M1 and M2-1 extracts. As example, for reaching an AAI of around 2.0 for LO, 100 mg/kg of M6 rosemary extract would be necessary,

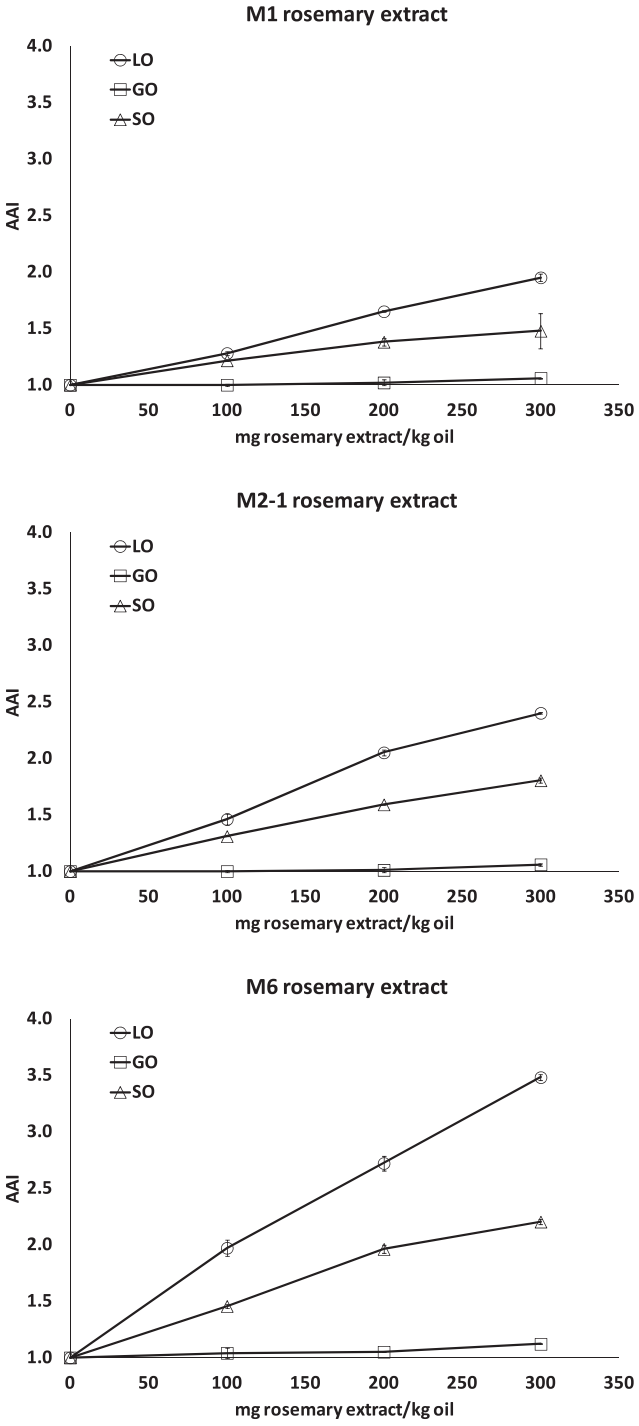
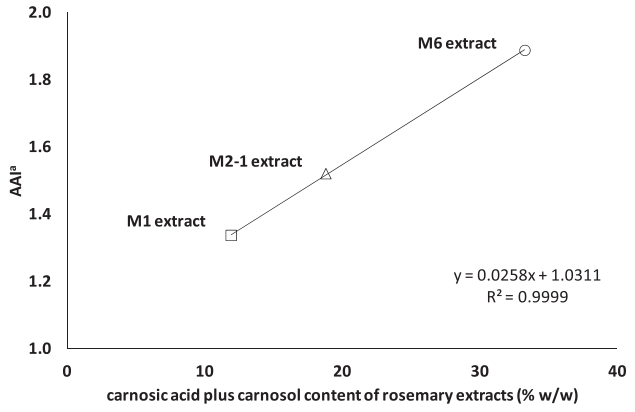


Fig. 1 Antioxidant activity index of selected rosemary extracts added at increasing concentrations to different vegetable oils and oxidized under Rancimat conditions.

compared to 200 mg/kg for the M2-1 extract or 300 mg/kg for the M1 extract (Fig. 1). In fact, 100 mg/kg of M6, 200 mg/kg of M2-1 and 300 mg/kg of M1 represent the addition of ca. 35 mg of carnosic acid plus carnosol per kg of oil,



[a] Average value at all the assayed concentrations and for the three oils

Fig. 2 Antioxidant power of supercritical rosemary extracts versus their antioxidant compounds (carnosic acid + carnosol) content.

which showed again that the antioxidant effect of a specific rosemary extract was mainly determined by the amount of antioxidant compounds added to the oil. Nevertheless, the amount of extract necessary to produce the desired antioxidant effectis an important factor to take into account for their useas antioxidants in oils, since their level of addition might affect aspects such as the own process of mixing/solubilization of the extract within the oil, or the inclusion of perceptible rosemary-aroma to oils. At this respect, the M6 extract showed the additional advantage of contributing with the lowest level of key volatile compounds. Nevertheless, further studies about the impact of this level of volatile compounds on the flavor attributes of oils should be elucidated. Concerning the effect of the rosemary extracts on the individual vegetable oils, interesting differences between oils were observed. Previously, it should be mentioned the different initial oxidative susceptibility of the evaluated oils in absence of any added antioxidant, the LO being the most labile, followed by GO, and the SO being the most stable vegetable oil (Table 1). It is frequently assumed that the oxidative susceptibility of lipids is higher as the proportion of PUFA increases[35]. Such effect was effectively observed in the present study since according to Table 1, the ratio PUFA/SAT of oils (LO > GO > SO) was in agreement with their initial oxidative stabilities (LO < GO < SO) in absence of rosemary extracts. However, this dependence of the oil oxidative susceptibility and fatty acid composition did not stand when the oil was oxidized in the presence of rosemary extracts. Thus, Tukey's posthoc test showed that the best protection against oxidation was evidence in the case of the most labile oil (LO), whereas an intermediate AAI was obtained for the most stable oil (SO) and the poorest antioxidant effect was observed for the medium stable oil (GO), regardless of the specific rosemary extract and the assayed concentration [p(oil) < 0.001] (Fig. 1). The specific composition in n-3 or n-6 PUFA more than the absolute level of double bonds might determine the particular oxidation of oils when rosemary extract was present. According to Table 1, LO mainly consisted of n-3 linolenic acid (53%) and lower levels of n-6 linoleic acid (19%), whereas n-6 linoleic acid was the major fatty acid of GO (68%) and was also important in SO (44%). Such increasing level of n-6 linoleic acid of oils as LO < SO < GO was found to be linearly correlated ($r^2 = 0.9984$) with the decreasing protective effect of rosemary extract as LO > SO > GO; whereas the level of n-3 linolenic acid did not seem to be responsible of such trend ($r^2 = 0.7404$). This result might suggest a limited antioxidant effect of rosemary extracts as the level of n-6 PUFA increased in oils, and it seemed that higher levels of addition would be necessary for evidencing a protective effect (Fig. 1). At this respect, Visioli et al.[36] concluded that the generation of oxidation products is not only related to the degree of unsaturation but also to the position of the double bonds. This statement was done when the oxidation of eicosapentaenoic acid (C20:5 n-3) and arachidonic acid (C20:4 n-6) was compared, and lower level of oxidation compounds were found for the former, despite its higher number of double bonds and, in turn, its higher expected oxidation. It might be reasonable to think that such implication of the location of double bounds in lipid oxidation might be also related to the easily of antioxidant compounds to exert their protective effect depending on the location of double bonds. However, previous information about the antioxidant effect of rosemary extracts in oils as affected by the location of double bonds in fatty acids has not been found, so further studies at this respect would be of interest to explain the observed effects.

On the other hand, the effect of the own endogenous antioxidants of the vegetable oils should be also involved in the particular differences found between oils when oxidized in presence of exogenous rosemary extracts. Constituents such as γ-tocopherol, lignans and sterols in LO or SO, or α-tocopherol and phenolic compounds in GO, have been pointed out as important antioxidants of these oils[37, 38]. Moreover, the interaction between endogenous antioxidant compounds of oils and those of rosemary extracts has been described by diverse authors, which might affect the potential activity of rosemary extracts, mainly by synergic effect in most cases[25, 18]. Such effects were not approached in the current assay in which the comparative ability of selected rosemary extracts as technological antioxidants of edible oils was the main aim. Nevertheless, further studies at this respect would be of interest, especially in the case of oils in which the assayed levels of rosemary extracts did not seem to be useful, such as GO.

4 CONCLUSIONS

Supercritical rosemary extracts were produced employing different extraction and fractionation conditions. Fractionation of the extract improved the antioxidant activity of one of the fractions collected, although process yield is reduced. Moreover, higher amounts of antioxidants seemed to be obtained when ethanol was employed as cosolvent.

The higher the reached content of antioxidants in the supercritical extracts the higher the antioxidant power in LO, GO and SO. Nevertheless, the magnitude of the protective effect was different depending on the individual oils. Indeed, the self-stability of the oil should influence the antioxidant effect of rosemary extract additives. In this respect, when rosemary extracts were added to the oil samples, the best successful protection was found for LO, that is for the most unstable oil. However, in the presence of supercritical rosemary extracts, higher antioxidant activity was found for SO than for GO, despite the fact that GO has a self-induction time almost three times lower than SO. This behavior was explained in our work in terms of the fatty acid composition of the different oils, and a possible limited antioxidant effect of supercritical rosemary extracts on n-6 PUFA, together with potential interactions with the own natural antioxidant compounds of the different oils. Nevertheless, this conclusion could not be generalized on the basis of the present study, and the potential use of supercritical rosemary extracts as antioxidants in vegetable oils should be tested for each particular application.

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