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ORIGINAL PAPER

Effect of the Addition of Membrane Processed Olive Mill Waste Water (OMWW) to Extra Virgin Olive Oil

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Abstract Modern membrane technologies are useful for enhancing the concentration of phenolic antioxidants in olive mill waste water (OMWW) to produce concentrates with valuable applications in functional foods. Three types of OMWW concentrates, each with different levels of solute concentration and purity, were obtained from a single OMWW batch and dissolved in two extra virgin olive oils to achieve saturated solutions. Three addition levels were considered. Accelerated aging testing of the oils was performed at 60 °C and the samples were analyzed after two, four, and 6 weeks of aging. D-optimal design was used to select the 26 experiments that allowed the evaluation of the influence of the different variables on oil stability. The addition of OMWW concentrates resulted in a significant increase in the Radical Scavenging Activity (RSA) of the olive oils. Under these mild experimental conditions, the moderate formation of fatty acid hydroperoxides was probably masked by interfering compounds.

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Introduction

Olive mill waste waters (OMWWs) are an unavoidable by-product of virgin olive oil production. The amounts produced, which are largely dependent on the technology employed, range between 50 and 80–110% of the initial olive weight with the traditional and the continuous processes, respectively [1]. Spain, Italy, and Greece account for nearly 80% of the global olive oil production; the season spans from October to March. Thus, in these countries, OMWW disposal is a significant environmental issue. The OMWW organic load is high, with a Biological Oxygen Demand (BOD) of up to 100 g L^{-1} and a Chemical Oxygen Demand (COD) of up to 200 g L^{-1} [2]. OMWWs are acidic, they contain sugars, organic acids, polyphenols, polyalcohols and proteins, have a mineral content of 1–2% (w/w) and contain 4–16% (w/w) organic matter [2]. The nitrogen, phosphorus, potassium and magnesium found in OMWW might be otherwise useful for crop fertilizers, except that the presence of phenolic compounds makes them toxic. While environmentally problematic, the antibacterial and antioxidant properties of these phenolic compounds make them interesting for health and nutritional applications. In particular, hydroxytyrosol and its esters, together with acetoxypinoresinol and pinoresinol (lignans) and oleocanthal, have interesting health properties [4]. Recently, OMWW membrane processing was proposed as an alternative to traditional physical–chemical, biological and thermal treatments [3, 5–7] with the objective of reducing environmental pollution while

simultaneously recovering OMWW useful by-products. Membrane processing is already extensively used within the food industry for the production of fruit juices, vegetable soups and milk [8]. Membrane cross-flow filtration is generally considered a non-intrusive and mild technology. Because it does not involve extreme chemical or physical conditions, such as very high temperatures, original molecular structures are preserved. OMWW treatment by integrated membrane processing allows the recovery of small volumes of concentrates containing the most valuable phenolics and other water soluble compounds, as well as a purified high-quality water side stream that can be re-used in the olive oil production process.

HIDROXI®, a commercial product obtained by extracting phenolic compounds from organic olives, is Generally Recognized as Safe (GRAS) and is considered to be a powerful and heat-stable antioxidant. Thus, in the context of functional food formulations, the addition of OMWW concentrates to food could enhance their levels of health promoting phenolics, although it is not known whether the presence of inorganic ions and other water soluble compounds interferes with their antioxidant properties; this bears further investigation. Starting from one single OMWW, the integration of different membrane processes allows production of different types of concentrates, with different physical–chemical properties, different phenolic content, and different Radical Scavenging Activities (RSA). The aim of this study was to investigate the effect of saturating two different extra virgin olive oils with three different types of OMWW concentrates. Accelerated aging tests of the oils were conducted at 60 °C, and the samples were analyzed after 2, 4, and 6 weeks of accelerated aging.

Experimental Procedures

Materials

Three types of OMWW concentrates were obtained by treatment of an OMWW produced at an olive mill factory located near Imperia (Italy) via integrated membrane processing. The techniques used to carry out micro-filtration (MF) and reverse osmosis (RO) were very similar: the feed was withdrawn from the plant reservoir and pumped over the surface of the membrane, which was housed in a stainless steel module, resulting in production of two streams, the retentate and the permeate. The former was recycled to the plant reservoir while the latter was continuously withdrawn until the desired Volume Concentration Ratio (VCR) was reached. VCR is defined as:

$$\mathrm{VCR} = V_0 / V_\mathrm{F} \quad (1)$$

where V_0 is the initial feed volume and V_F is the final concentrate volume.

The process included a first micro-filtration (MF) stage operating at 3 bar and 40 °C, in which vegetation water was passed through a porous ceramic membrane. The resulting clarified micro-filtered stream was subjected to a successive reverse osmosis (RO) step (operating at 30 bar and 25 °C) to obtain a concentrate (W_A) with a high content of dissolved substances. The ceramic membrane (P19-40 Pall, Port Washington, NY) was a multi-channel element (19 channels, length 850 mm, 4 mm diameter) made of α-alumina with a mean pore size of 0.2 μm. The RO membrane (Filmtec Dow, Midland, MI) was a spirally wound element (SW30-4040, length 40″, diameter 4″) made of a selective polyamide with a NaCl retention $R = 99.4\%$. A second type of OMWW concentrate (W_B) was obtained by a further RO concentration (50 bar, 25 °C) of W_A up to a Volume Concentration Ratio 2 (VCR).

One thousand (1,000) L of OMWW were micro-filtered through a module containing 7 MF ceramic membranes in series. After a 7 h filtration time, 800 L of permeate were obtained. The permeate was then passed through a module containing 1 RO polyamide membrane in order to obtain, after 5 h operating time at 30 bar and 25 °C, 200 L of W_A concentrate, which was further treated at higher pressure (50 bar) to produce, after 7 h operating time, 100 L of W_B concentrate. The longer time for the second RO step was due to the strong decline of the permeate flux of the RO membrane caused by the progressive increase of the osmotic pressure of the concentrated feed.

The third type of OMWW concentrate (W_C) was obtained from W_A after its purification by ionic exchange resins (1:2 anionic/cationic ratio; 1:2 resins/feed ratio).

Two samples of extra virgin olive oil were considered: the first was obtained by a continuous two-phase olive mill (L), the second by a continuous three-phase system (M).

Chemicals

1,1-diphenyl-2-picrylhydrazyl radical (DPPH•) was supplied by SIGMA Chemie (Germany), and syringic acid was purchased from Fluka Chemie GmbH (Buchs, Switzerland). External standardization was used for ion chromatography and chloride and sulfate standards were supplied by Dionex (Sunnyvale, CA); phosphate standard was supplied by Ultra scientific (Bologna, Italy). Solvents used were analytical, HPLC or spectroscopic grade and were supplied by Merck (Darmstadt, Germany). Eighteen micro ohm deionized water from a Millipore (Billerica, Massachusetts, USA) Milli-Q water purification system was used to prepare the chromatographic mobile phase.

Samples

In order to saturate the oils, the OMWW W_A, W_B and W_C concentrates were carefully added to the two virgin olive oils (L and M) at a 0.1 mL/L concentration. These samples were then gently shaken by vortex for 10 min. A further dilution with L and M resulted in oil samples containing 0.05 mL/L OMWW. The selected aging times were 2, 4, and 6 weeks.

Accelerated Aging Test

Accelerated aging testing of the oil samples was performed following AOCS Recommended Practice [9]. The relevant samples were divided into 10-g sub-samples in closed amber glass 20-mL bottles. The area exposed to air was 0.80 cm^2/g. The 20-mL bottles were heated to 60 °C in a forced draft oven for 2, 4 and 6 weeks.

Physical–Chemical Analysis of the OMWWs

The pH was measured with a HANNA pH209 pH meter (Hanna Instruments, Woonsocket, RI., USA) and conductivity with a HANNA HI9033 multi-range conductivity meter. Total dissolved solids and organic residue were determined as recommended by the American Public Health Association [10].

Cl^-, PO_4^{3-} and SO_4^{2-} anions were determined by a Dionex DX-120 ionic chromatograph equipped with an Ion Pac® AG9-HC 4 × 50 mm pre-column, an Ion Pac® AS9-HC 4 × 250 mm column and a conductivity detector, at 1 mL/min column flow and 25 °C column temperature. The isocratic mobile phase was a 9 mM Na_2CO_3 solution. Quantification was obtained using external standard solutions containing Cl^-, PO_4^{3-} and SO_4^{2-} ions in known amounts and obtained by dilution of the standard solutions.

The total phenol content was determined using Folin–Ciocalteau reagent [11] and was expressed as Gallic Acid Equivalent (GAE).

RSA of the OMWWs

Exactly 1 mL of the OMWW concentrate was transferred into a 10-mL volumetric flask and methanol added to the mark. The resulting suspension was sonicated for 1 min and centrifuged at 3,500 rpm for 10 min. The clear solution was used to prepare methanolic solutions at decreasing concentrations, which were employed to determine the antioxidant activity of OMWWs analyzed by reaction with a 10^{-4} M solution of DPPH• [12]. The absorbance of the reaction mixture was read after 60 min, at the steady state, at 515 nm, and the antioxidant activity of 1 mL OMWW was expressed as $mmol_{DPPH•}$ equivalent. Three replicate analyses were performed for each W sample.

Free Acidity, Peroxide Value, Fatty Acid Composition, UV Absorbance and Minor Polar Compounds (MPCs) of the Oil Samples

Except for the analysis of MPCs, the analytical methods described in European Regulation EEC 2568/91 [13] and later amendments were used. Two replicates were performed for each analysis and each test sample. MPCs were extracted from 1 g of crude oil by a mixture of water and methanol 20:80 vol/vol after the addition of the internal standard (syringic acid). The identification and quantification of MPCs was carried out by reverse phase HPLC [14] on a Spherisorb ODS2 column (250 × 4.6 mm), gradient elution (solvent A: water + 0.2% phosphoric acid; solvent B: methanol: acetonitrile 50:50 by vol) at a 1 mL min flow rate, and Diode Array Detection (DAD) [14]. MPCs were determined only once.

RSA of Oil Samples

The oil's ability to scavenge the stable DPPH• radical, was determined as recently reported [15]. One gram of oil was dissolved in ethyl acetate in a 10-mL volumetric flask; 1 mL of this solution was transferred into a second 10-mL volumetric flask and a daily prepared DPPH• mother solution (approximately 10^{-4} M in ethyl acetate) was added to the mark. The reaction flask was shaken for 10 s in a Vortex apparatus and was allowed to stand in the dark for 30 min. The residual absorbance was measured at 515 nm against a blank solution (without radical). The initial DPPH• concentration was measured by control samples (without oils), obtained by the dilution of 1 mL of ethyl acetate by the DPPH• mother solution.

The RSA of the samples was expressed as the % reduction of DPPH• concentration in a DPPH• solution exactly 1.00×10^{-4} M and was not dependent on the concentration of the daily DPPH• solutions

$$\mathrm{RSA} = ([\mathrm{DPPH•}]_{\mathrm{control}} - [\mathrm{DPPH•}]_{\mathrm{sample}})/10^{-4} \times 100 \quad (2)$$

Three replicate analyses were performed for each sample.

Experimental Design and Statistical Analysis

Experimental design and statistical analysis were performed by using Matlab 4.2 [16] routines written by one of the authors. The Response Surface Methodology (RSM) was used to study the effects of the experimental variables

Table 1 Physical chemical parameters of the OMWW concentrates

OMWW concentrate	pH	Conductivity mS/cm	120 °C Residue g/L	600 °C Residue g/L	Cl^- mg/L	PO_4^{3-} mg/L	SO_4^{2-} mg/L	RSA mmol/mL	Total phenol GAE/kg	DHPEA mg/L	HPEA mg/L
W_A	4.92 ± 0.05	26.0	172.18	38.10	4,040 ± 2	2,310 ± 5	635 ± 1	$3.08 \pm 0.10 \times 10^{-1}$	16.9 ± 0.4	1,936	625
W_B	4.80 ± 0.04	28.0	217.60	80.50	4,980 ± 3	3,200 ± 2	810 ± 1	$2.82 \pm 0.11 \times 10^{-1}$	19.8 ± 0.5	1,845	595
W_C	2.98 ± 0.04	6.0	99.65	6.64	95 ± 3	n.d.	60 ± 1	$1.14 \pm 0.15 \times 10^{-1}$	9.4 ± 0.3	1,354	398

GAE gallic acid equivalent, *DHPEA* dihydroxyphenylethanol (hydroxytyrosol), *HPEA* 4-hydroxyphenylethanol (tyrosol), *n.d.* not detectable

on the stability of the oils, which was evaluated by several response variables, i.e. UV absorbance at 232 and 270 nm, oleic/palmitic, linoleic/palmitic, linolenic/palmitic acid ratios and RSA.

As far as MPCs are concerned, a mathematical model was built for each free and esterified MPC, which were considered as response variables together with some particular ratios (i.e. tyrosol/oleocanthal). The total MPC content was not separately studied, since it was highly correlated to the single MPC content.

Table 2 The experimental plan

Sample	Oil	W amount μL/L	Weeks at 60 °C	OMWW
1	M	50	6	W_C
2	L	100	4	W_A
3	M	0	6	W_A
4	M	50	2	W_B
5	L	100	6	W_B
6	L	0	2	W_B
7	M	100	6	W_A
8	L	100	2	W_C
9	M	0	6	W_C
10	M	0	4	W_B
11	L	100	6	W_C
12	M	100	6	W_B
13	M	0	2	W_A
14	M	100	2	W_A
15	L	0	4	W_A
16	M	100	2	W_C
17	M	50	4	W_B
18	L	100	2	W_B
19	M	100	4	W_C
20	L	50	6	W_A
21	L	0	2	W_C
22	M	0	2	W_C
23	L	0	6	W_B
24	L	50	4	W_C
25	L	50	2	W_A
26	L	0	6	W_C

For each determination, the order of sample analysis was randomized.

Results and Discussion

Table 1 reports the physical–chemical parameters detected in the different Ws and allows some preliminary considerations in the context of the influence of the different treatments. The increase of conductivity and ion content produced by the concentration of W_A to W_B by RO was lower then what had been predicted on the basis of VCR; moreover, the small increase of the 120 °C residue results from a loss of organic matter in permeate flux, which was indirectly confirmed by the lower DPPH• equivalent amount. As far as W_C is concerned, preliminary experiments led to the use of resins in a 1:2 anionic/cationic ratio and in a 1:2 resin/feed ratio. Under these conditions a strong effect on the ion content of W_C was observed but the 120 °C residue of W_C and its RSA showed that part of the organic solutes was also retained.

The analysis of the MPC content of OMWW concentrates was performed by HPLC–DAD under the same conditions used for the HPLC analysis of MPCs in oils [14]. 2,4-dihydroxyphenyl ethanol (DHPEA or hydroxytyrosol) and 4-hydroxyphenyl ethanol (HPEA or tyrosol) were the major free phenolic compounds and their amounts were $W_A > W_B > W_C$, in the ranges between 1,845–1,354 and 398–595 mg/L for DHPEA and HPEA, respectively. The amounts of linked phenolic compounds were practically non-significant. It is interesting to note that in spite of the further concentration step for W_B, the content of the two phenolic compounds was higher in W_A than in W_B: this finding confirms the loss of organic matter in the permeate flux and is in accordance with the RSA of the OMWWs.

As far as the two extra-virgin olive oils are concerned, the total MPC content as determined by HPLC was 91.0 and 108.0 mg/kg (expressed as tyrosol) for oils M and L respectively, with free DHPEA and HPEA contents close to 10% of total MPCs. Two secoiridoid precursors of DHPEA, i.e. an isomer of the oleoeuropeine aglycone (17.2 and 18.1 mg/kg) and the dialdehydic form of elenolic acid

linked to DHPEA [17] (10.4 and 11.1 mg/kg) were also detected, together with the two lignans pinoresinol (2.7 and 3.3 mg/kg) and 1-acetoxypinoresinol (8.8 and 23.3 mg/kg) [18], the dialdehydic form of elenolic acid linked to HPEA (oleocanthal) [17] (20.6 and 19.4 mg/kg) and other minor secoiridoid derivatives.

The study of the influence of OMWW addition on oil stability was performed at 60 °C for 6 weeks. The experiment was conducted in the dark in a forced draft oven. The 60 °C temperature was chosen since the mechanism of oxidation at 60–80 °C is the same as oxidation at room temperature [19]. The duration of the experiment was defined on the basis of the study published by Mancebo-Campos et al. [20], which showed a rapid development of oxidation of virgin olive oils at 60 °C.

The stability to oxidation of treated and non-treated virgin olive oils was evaluated by UV absorbances at 232 nm (primary oxidation products) and 270 nm (secondary oxidation products) and by the ratios between the major unoxidized unsaturated fatty acids (oleic, linoleic and linolenic) and saturated palmitic acid [20]. RSA and its evolution with aging was used to evaluate the stability of antioxidant compounds added with OMWWs.

In order to study the influence of two qualitative variables (oil and type of concentrated OMWW) and two quantitative variables (amount of concentrated OMWW and aging time), at three different levels (0, 50, 100 μL/L oil and 2, 4 and 6 weeks, respectively), a D-optimal design was employed. This type of design looks for the subset of experimental points leading to the highest ratio between the information obtained and the experimental effort required. Among the 54 possible experiments (2 types of oil * 3 amounts of added W * 3 aging times * 3 types of W), the D-optimal design selected the 26 experiments reported in Table 2.

The four independent variables were coded as follows:

- oil type (variable X_1): M = −1, L = +1;
- amount of added W (Variable X_2): 0 μL/L= −1, 50 μL/L = 0, 100 μL/L = +1;
- aging time (variable X_3): 2 weeks = −1, 4 weeks = 0, 6 weeks = +1;
- W (variable X_4): W_A = [1 0]; W_B = [0 1]; W_C = [0 0] (since it is a qualitative variable at more than two levels, in the model matrix as many columns as levels are required, minus 1)

The selected experiments allowed the estimation of the coefficients of the following model:

$$\begin{aligned} Y = {} & b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{4A}X_{4A} \\ & + b_{4B}X_{4B} + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \\ & + b_{22}X_2^2 + b_{33}X_3^2 \end{aligned} \quad (3)$$

where b_0 is the constant, b_1, b_2 and b_3 are the linear terms of variables X_1, X_2 and X_3, b_{12}, b_{13} and b_{23} are the interaction terms and b_{22} and b_{33} are the quadratic terms of the two quantitative variables.

However, the interpretation of coefficients b_{4A} and b_{4B} is somewhat more complex. As previously stated, the qualitative variable W has three levels, and therefore it needs two terms, whose values are the estimate of the difference between the response obtained when the variable has the corresponding level (A or B, respectively) and the response obtained when the variable has the "implicit" level (C).

Except for MPCs, each point of the experimental plan was performed twice, for a total of 52 experiments.

The models computed for the fatty acid ratios were significantly influenced by oil type (X_1) alone. For example, the model obtained for oleic/palmitic acid ratio was

$$\begin{aligned} 18:1/16:0 = {} & 5.89 - \mathbf{0.34\,X_1}(***) \\ & + 0.01X_2 - 0.04\,X_3 - 0.05\,X_{4A} \\ & + 0.01\,X_{4B} - 0.04\,X_1X_2 \\ & + 0.05\,X_1X_3 - 0.00\,X_2X_3 \\ & + 0.09\,X_2^2 - 0.03\,X_3^2 \end{aligned} \quad (4)$$

In order to exclude a possible masking effect of the oil, the two groups of samples obtained from the same oil were considered separately, but the 6 models obtained confirmed that none of the coefficients of the model were significant ($p < 0.05$) on the fatty acid ratios, in contrast to the findings of Mancebo et al. [20] for the aging weeks.

Similarly, the UV absorbance at 232 nm, which measures for the presence of hydroperoxides, did not appear significantly influenced ($p < 0.05$) by aging or by the concentrated OMWWs, but in this case the Relative Standard Deviation (RSD) of the two replicates was quite high, probably reflecting the presence of interfering compounds that might have masked the regular increase of hydroperoxides. On the contrary, the model of the UV absorbance at 270 nm, which is related to the formation of secondary oxidation products, was most significantly influenced ($p < 0.001$) by aging time rather than by the oil. The quadratic effect of X_2, though statistically significant, was so small that it could be ignored.

$$\begin{aligned} K_{270} = {} & 0.263 - \mathbf{0.021\,X_1}(***) + 0.000\,X_2 \\ & + \mathbf{0.052\,X_3}(***) + 0.005\,X_{4A} \\ & + 0.003\,X_{4B} - 0.002\,X_1X_2 - 0.003\,X_1X_3 \\ & + 0.002\,X_2X_3 + \mathbf{0.010\,X_2^2}(*) - 0.006\,X_3^2 \end{aligned} \quad (5)$$

The high coefficient of X_3 showed that K_{270} increased rapidly with time, but, in accordance with a previous study [20], it did not reach a plateau, since the quadratic X_3 term was not significant. When compared to L samples, M

Table 3 Physical chemical parameters of the raw extra virgin olive oils

Extra virgin olive oil	Acidity	Peroxide value	UV parameters			Fatty acid composition								
			K270	K232	ΔK	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3	20:0	20:1
L	0.33 ± 0.02	7.7 ± 0.2	0.150 ± 0.007	1.83 ± 0.07	−0.001 ± 0.000	13.21 ± 0.04	1.09 ± 0.01	0.06 ± 0.00	0.12 ± 0.00	2.36 ± 0.03	74.30 ± 0.16	7.47 ± 0.02	0.64 ± 0.01	0.35 ± 0.01	0.25 ± 0.02
M	0.29 ± 0.02	9.3 ± 0.3	0.164 ± 0.000	1.83 ± 0.03	−0.001 ± 0.000	11.86 ± 0.12	0.95 ± 0.01	0.08 ± 0.01	0.14 ± 0.01	2.77 ± 0.01	75.52 ± 0.05	7.25 ± 0.01	0.63 ± 0.00	0.41 ± 0.01	0.26 ± 0.03

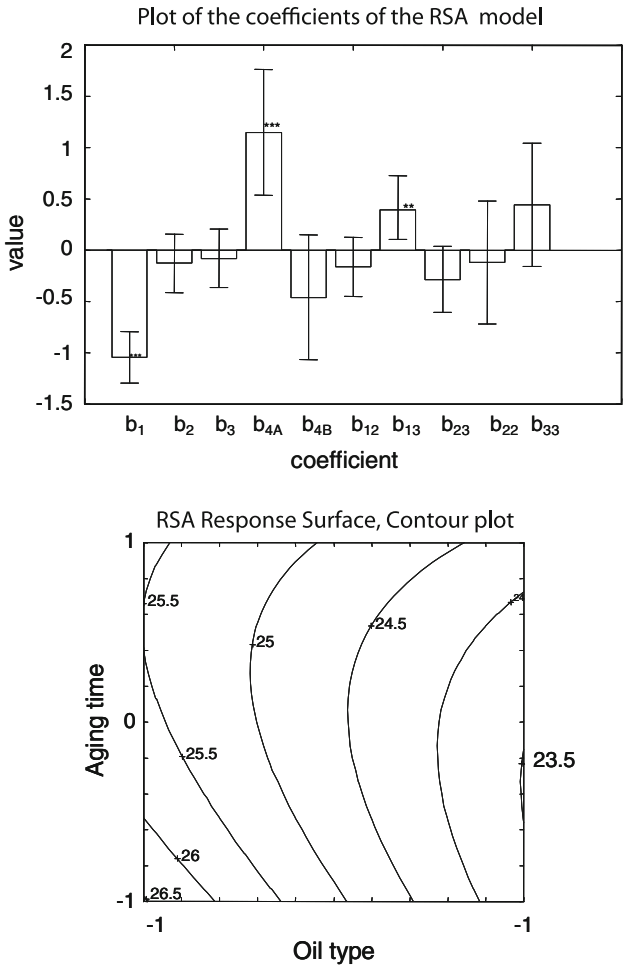

Fig. 1 Plots of the coefficients of the models and of the response surface of the RSA computed for oil samples (**$p < 0.01$, ***$p < 0.001$)

samples showed higher K_{270} values that can be related to the worse oxidative condition of the crude oil supported by its higher peroxide value (Table 3) at the beginning of the experiments. The obtained K_{270} model had high predictive ability, with a 91.0% variance explained in cross validation. By comparing the 0.016 Root Mean Square Error in Cross Validation (RMSECV) of this model to the 0.020 pooled standard deviation of the analytical experiments it can be concluded that a further improvement of the predictive ability of the model is impossible.

The equation of the RSA model, whose coefficients are visualized in Fig. 1 together with the Response Surface contour plot, shows that the type of W had a significant effect on this response variable:

$$\begin{aligned} \text{RSA} = {} & 23.43 - \mathbf{1.04\,X_1}(***) - 0.13\ X_2 0.08\ X_3 \\ & + \mathbf{1.15\,X_{4A}}(***) - 0.46\ X_{4B} - 0.16\ X_1X_2 \\ & + \mathbf{0.39\,X_1X_3}(**) - 0.29\ X_2X_3 \\ & - 0.12\ X_2^2 + 0.44\ X_3^2 \end{aligned} \quad (6)$$

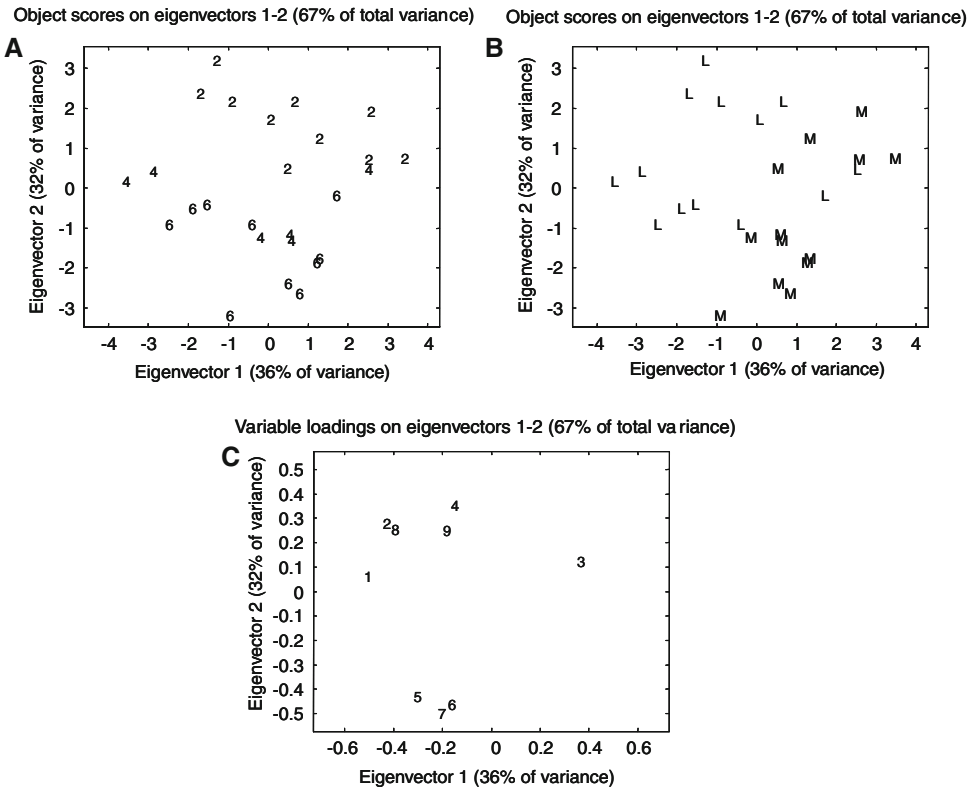

Fig. 2 Score (**a** and **b**) and Loading (**c**) plots obtained by the PCA of the data obtained from the MPC analysis of 26 samples of added oils. Scores are coded according to aging time (2, 4 and 6 weeks, graph A) and oil type (L and M, graph B). Loadings are coded according to the list reported in "Result and Discussion"

The RSA of M samples was significantly ($p < 0.001$) higher, and the addition of concentrated OMWWs strongly affected the RSA of sample oils. In particular, the addition of W_A significantly ($p < 0.001$) increased the RSA with respect to W_B and W_C (implicit coefficient), thus confirming that the second RO step on OMWWs had decreased their RSA.

The Response Surface contour plot of RSA also allows visualizing the interaction of X_1 (oil type) and X_3 (aging time). The plot shows that in the case of oil M, the response decreased with time, while with oil L, time had no effect. On the other hand, the difference between the two oils decreased with time.

As far as MPC analysis is concerned, Principal Component Analysis (PCA) was applied to the data obtained from the analysis of 26 samples of added oils, each sample being described by nine variables (1, DHPEA; 2, HPEA; 3, *p*-coumaric acid; 4, dialdehydic form of elenolic acid linked to DHPEA, 5, oxidized dialdehydic form of elenolic acid linked to DHPEA, 6, dialdehydic form of elenolic acid linked to HPEA, 7, pinoresinol, 8, 1-acetoxypinoresinol, 9, oleoeuropeine aglycone).

Figure 2 shows two discriminating directions. Figure 2a and c show that variables 5, 6, and 7 discriminate the samples according to their aging, while Fig. 2b and c show that variables 1, 2, 4, 8, and 9 discriminate the samples according to the oil type.

Subsequently, mathematical models built for each MPC as a function of the studied variables confirmed the influence of the oil type on MPC content and showed that the amount of free and esterified phenols was also generally affected by aging time (data not reported). Similar results were obtained considering some ratios between the amounts of free and esterified MPCs, such as tyrosol/oleocanthal ratio (Fig. 3), as response variables. The negative and significant ($p < 0.05$) coefficient of X_2X_3 term showed that the added OMWWs contributed to decrease this ratio when aging time increased. An unexpected result was that the amounts of DHPEA and HPEA were not significantly influenced by the addition of the three tested Ws in spite of their high content of these phenols, which was particularly significant in W_B. W_s volume added to oils was probably too small, since oil saturation was immediately reached.

Conclusions

The results obtained indicate that the addition of concentrated OMWWs does not significantly influence the oxidative stability of the two oils in question. Nevertheless, in the temperature/time conditions under study, oxidation was quite slow and the involvement of unsaturated fatty acids in

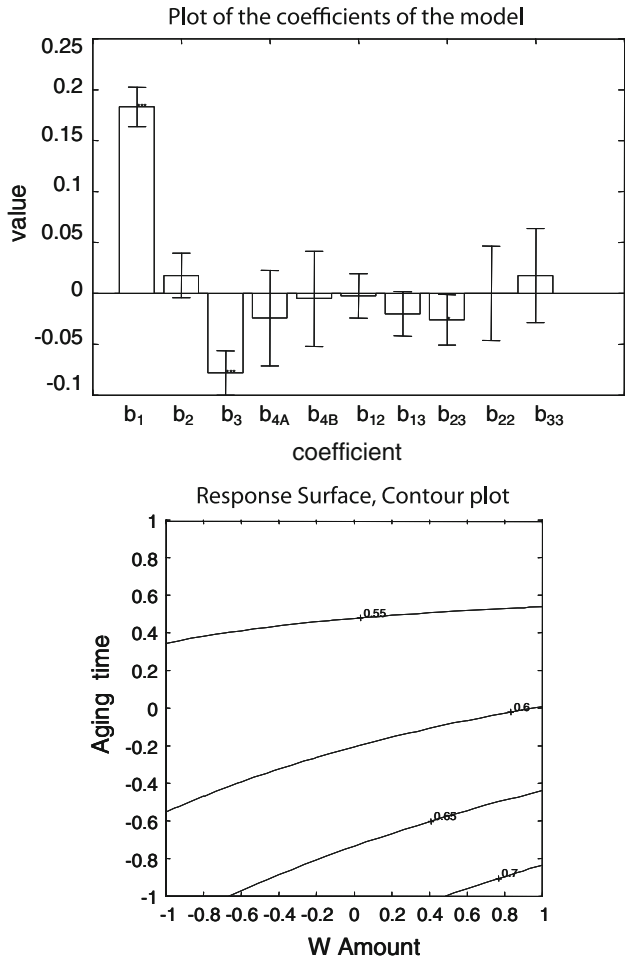

Fig. 3 Plots of the coefficients of the models and of the response surface of the tyrosol/oleocanthal ratio computed for oil samples (*$p < 0.05$, ***$p < 0.001$)

the radical reaction was detectable neither by the ratios between unsaturated fatty acids and palmitic acid nor by the absorbance of their hydroperoxides at 232 nm. On the basis of these results, it is possible to assume that in the range of experimental conditions tested, the development of oxidation was limited to the decomposition of existing hydroperoxides, and that the addition of the concentrated OMWWs in the amounts tested had no effect on these reactions since their *ortho*-diphenolic content has only a radical scavenging activity that cannot prevent the decomposition of the primary oxidation products.

The significant and lasting observed effect of OMWWs on oil RSA is particularly promising in light of functional food formulation and offers an excellent prospect for OMWW exploitation. However, the radical scavenging activity of the added oil samples was not increased by the second RO step nor by decreasing the ion content of the added waste water. It is possible that the small amounts of OMWW concentrates employed did not allow expression of different levels of protective activities among the different tested waters. Thus, the development of innovative formulations that allow incorporation of higher amounts of OMWW concentrates in oils in order to take full advantage of their high content of phenolic antioxidants appears to be very promising.

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