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Antioxidant Activity of Oregano, Parsley, and Olive Mill Wastewaters in Bulk Oils and Oil-in-Water Emulsions Enriched in Fish Oil

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The antioxidant activity of oregano, parsley, olive mill wastewaters (OMWW), Trolox, and ethylenediaminetetraacetic acid (EDTA) was evaluated in bulk oils and oil-in-water (o/w) emulsions enriched with 5% tuna oil by monitoring the formation of hydroperoxides, hexanal, and *t*-*t*-2,4-heptadienal in samples stored at 37 °C for 14 days. In bulk oil, the order of antioxidant activity was, in decreasing order ($p < 0.05$), OMWW > oregano > parsley > EDTA > Trolox. The antioxidant activity in o/w emulsion followed the same order except that EDTA was as efficient an antioxidant as OMWW. In addition, the total phenolic content, the radical scavenging properties, the reducing capacity, and the iron chelating activity of OMWW, parsley, and oregano extracts were determined by the Folin−Ciocalteau, oxygen radical absorbance capacity, ferric reducing antioxidant power, and iron(II) chelating activity assays, respectively. The antioxidant activity of OMWW, parsley, and oregano in food systems was related to their total phenolic content and radical scavenging capacity but not to their ability to chelate iron in vitro. OMWW was identified as a promising source of antioxidants to retard lipid oxidation in fish oil-enriched food products.

KEYWORDS: Lipid oxidation; fish oil; long-chain polyunsaturated fatty acids; olive mill wastewater; oregano; parsley; antioxidant

INTRODUCTION

Fish oil is a source of long-chain polyunsaturated fatty acids (LC-PUFA), which have shown several positive effects for human health (*1–3*). As a consequence, there is an interest in enriching food products with fish oil to increase the consumption of health-promoting lipids. However, the use of fish oils to enrich food products is limited due to their high susceptibility to oxidation. Lipid oxidation in food affects its sensory properties, through development of off-flavors, and reduces its nutritional value. Lipid oxidation is affected by several parameters including the degree of unsaturation of the lipid, temperature, light, the level of antioxidants, and the presence of transition metals (*4*). Metals such as iron and copper are found in most foods since they are common constituents of raw food materials, water, ingredients, and packaging materials (*5, 6*). It is generally accepted that lipid hydroperoxides, primary lipid oxidation products, decompose by a metal-catalyzed reaction to yield volatile compounds that are responsible for off-flavors in oxidized foods (*7*). The physical properties of food also greatly influence lipid oxidation. For example, oxidation in bulk oil and in oil-in-water (o/w) emulsion differs at a mechanistic level due to the properties of the emulsion droplet interface and the ability of components, including pro-oxidants, antioxidants, and oxidizable substrates, to partition differently into the oil or water phases of the emulsion or to concentrate at the interface (*8, 9*).

There is a long history of the use of antioxidants to retard lipid oxidation in food (*10*). The effectiveness of an antioxidant in food depends on several factors including its chemical reactivity (e.g., toward radical scavenging and metal chelation), interaction with other food components, the environmental conditions (e.g., pH), and the physical location of the antioxidant in different food systems (e.g., bulk oil and o/w emulsions) (*11*). The empirical observation that hydrophilic antioxidants are more active in bulk oil systems whereas lipophilic antioxidants are more active in o/w emulsions is referred to as the "polar paradox". This phenomenon is explained by the location of polar antioxidants in the air−oil interface in bulk oil and nonpolar antioxidants in the oil−water interface in o/w emulsions (*7*). It has been suggested that oxidative reactions in bulk oil could be more prevalent at the air−oil interface, whereas it is assumed that lipid oxidation is more prevalent at the oil−water interface in o/w emulsions (*7*). However, several studies found that the polar paradox hypothesis seems to be too simple to explain the

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complex mechanism of antioxidant efficacy in multiphase systems (*12, 13*).

As a consequence of consumer preference for natural ingredients and concerns about possible toxic effects of synthetic antioxidants (*14*), there is growing interest in the use of natural antioxidants. Phenolic compounds are ubiquitous in the plant kingdom and are known to exhibit antioxidant properties by terminating radical chains and binding metal catalysts (*7*). Three natural sources of phenolic compounds are olive mill wastewaters (OMWW), oregano, and parsley. OMWW, a byproduct of olive oil production, represent a particularly rich source of phenolic compounds, the most abundant of which is hydroxytyrosol (*15*). The antioxidant activity of hydroxytyrosol is well-established (*16–19*). The use of OMWW as a food antioxidant has been reported only in a few studies (*20, 21*), and to our knowledge, none of these concern its use to stabilize products containing LC-PUFA. The herb oregano (*Origanum vulgare*) is widely used in Mediterranean cookery. It has a broad composition of different types of phenolic compounds including phenolic acids (rosmarinic acid, caffeic acid, *p*-coumaric acid, and caffeoyl derivatives), flavones (apigenin and luteolin), and flavanols (myricetin and quercetin) (*22–24*). Dry oregano and organic solvent extracts have been shown to exhibit remarkable antioxidant activity in various fats and oils including lard, sunflower oil, mackerel oil, and menhaden oil (*25–30*). The antioxidant properties of parsley (*Petroselinum crispum*) have also been attributed to its phenol content (*31*), particularly caffeic acid, in which it is very rich (*22, 24*). Little is known about the parsley antioxidant activity in foods enriched with fish oil (*32*).

The objective of this study was to evaluate the capacities of OMWW, oregano, and parsley to retard lipid oxidation in model foods containing 5% tuna oil and 3.5 mg/kg added Fe^{2+}. In addition, two synthetic antioxidants, ethylenediaminetetraacetic acid (EDTA) and Trolox, were evaluated. EDTA is a very effective metal chelator and has been widely used to prevent lipid oxidation in food emulsions such as mayonnaise, salad dressing, and milk (*33–37*). Trolox, a water-soluble α-tocopherol analogue, is widely used as a reference antioxidant for in vitro studies, and its antioxidant activity in food has been studied previously (*38, 39*). To investigate the influence of physical properties on antioxidant activity, the stability of 5% tuna oil to oxidation both in bulk oil and in o/w emulsion was evaluated in the presence and absence of antioxidants. The oxygen radical absorbance capacity (ORAC assay), ferric reducing antioxidant power (FRAP assay), and metal chelating capacity [iron(II) chelating activity assay, ICA] of OMWW, oregano, and parsley were assessed to provide additional information about their mechanisms of action.

MATERIALS AND METHODS

Chemicals. All chemicals used were of analytical grade. Hexanal, (±)-6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, fluorescein, EDTA, anhydrous iron(II) chloride (beads), and potassium iodide were obtained from Sigma-Aldrich (Buchs, Switzerland). Folin−Ciocalteu (FC) reagent, Tween 20, and sodium thiosulphate solution were purchased from Merck (Dietikon, Switzerland). 2,2′-Azobis(2-amidino propane) dihydrochloride (AAPH) was purchased from Wako Chemicals GmbH (Germany). Randomly methylated-β-cyclodextrin (RMCD) was purchased from Cyclolab Ltd. (Budapest, Hungary). Deuterium-labeled hexanal-d_{12} was obtained from CDN Isotopes Inc. (Canada). Hydroxytyrosol was purchased from Extrasynthese (Genay, France). Medium-chain triglycerides (MCT) oil was purchased from Cognis GmbH & Co. (Germany). Tuna oil was provided by Sofinol S.A (Manno, Switzerland). Lyophilized oregano and parsley were purchased from a local supplier (Lausanne, Switzerland), and lyophilized OMWW were provided by Ebiser (Castellón, Spain). Oregano, parsley, and OMMW were stored until analyses in a dissecator containing silica gel as the drying agent.

Preparation of Emulsions and Bulk Oils. The o/w emulsion contained acetate buffer, pH 3.6 (78.4% w/w), MCT oil (15% w/w), tuna oil (5% w/w), Tween 20 (0.5% w/w), and $FeCl_2$ solution (0.1% w/w, 3.5 mg/kg Fe^{2+} final concentration). Ground oregano, parsley, and OMWW (particle size ≤ 200 μm) were added at 1% w/w, whereas EDTA and Trolox were added at a level of 0.0075% w/w (75 mg/kg). The emulsion was prepared by mixing all of the ingredients with a polytron (Kinematica Inc., Switzerland) and homogenizing at 500 bar in a microfluidizer processor (Microfluidics Corp. Newton, United States). After homogenization, antioxidants were added to the emulsion and mixed using the polytron. The pH of all of the emulsions ranged between 3.6 and 3.8. Bulk oil comprised MCT oil (93.9% w/w), tuna oil (5% w/w), and $FeCl_2$ solution (0.1% w/w, 3.5 mg/kg Fe^{2+} final concentration). Antioxidants were added at the same concentrations as detailed above for the emulsions.

Storage Study. Duplicate samples of o/w emulsion and bulk oil were stored in the dark at 37 °C for 14 days. Hydroperoxides, volatile compounds (hexanal and *t*,*t*-2,4-heptadienal), and emulsion droplet particle sizes were determined regularly (every 2/3 days) during the storage period. For hydroperoxides and particle size determinations, several sample aliquots (10 g) were transferred into 20 mL headspace vials, sealed, and stored under the conditions described above. For the determination of volatile compounds, the same procedure was performed except that only 1 g of sample aliquot was transferred into the headspace vials. Sample aliquots were discarded after each analysis. The antioxidant activity was expressed as the % inhibition in the formation of oxidation products (hydroperoxide and volatile compounds) in the sample containing the antioxidant (aox) with respect to the control at the end of the storage study (14 days):

$$\%\ \text{inhibition} = \left(1 - \frac{\text{oxidation products aox}_{\text{day 14}}}{\text{oxidation products control}_{\text{day 14}}}\right) \times 100$$

Determination of Fatty Acid Profile. The fatty acid composition of tuna oil was determined by gas chromatography (GC) after transesterification to fatty acid methyl esters. Tuna oil was transesterified in the presence of methanolic potassium hydroxide solution according to Muuse et al. (*40*) and ISO standard 15884 (*41*). Fatty acid methyl esters were analyzed by GC using the conditions described previously for the separation and quantification of fatty acid methyl esters (*42*).

Determination of Volatile Compounds by Head Space Solid-Phase Microextraction−Gas Chromatography−mass Spectrometry (HS-SPME-GC/MS). Volatile compounds were determined by HS-SPME-GC/MS as described previously (*43*). Briefly, the SPME employed a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber: equilibration temperature, 37 °C; equilibration time, 45 min; and desorption time, 1 min. GC/MS analyses were performed on a GC Top8000 equipped with a PAL SPME injector and coupled to a single quadrupole Voyager spectrometer (Brechbuehler AG, Zurich, Switzerland). The GC splitless injector was heated at 220 °C. Helium was used as carrier gas in constant pressure mode at 100 KPa. The injector split was set at 30 mL/min, and the valve was opened after 1.5 min. A DB-5MS capillary column (60 m × 0.25 mm i.d.; film thickness, 0.25 μm; Milian, Geneva, Switzerland) was used. The column temperature was held at 35 °C for 8 min, increased to 120° at 5 °C/min, held for a further 1 min, then increased to 250 °C for 11 min at 10 °C/min. The temperatures of the ion source and transfer line were 200 and 225 °C, respectively. Electron ionization mass spectra were acquired at 70 eV from 35 to 250 *m*/*z* in full scan mode (2 scans/s). Volatiles were identified both by MS library searches and by comparison of retention times (t_R) with authentic external standards when available. Hexanal-d_{12} (1 mg/kg) was added as an internal standard prior to analyses. The volatile compounds were determined in relative terms as the ratio between the volatile peak area with regard to the hexanal-d_{12} peak area.

Determination of Lipid Hydroperoxide Levels. Lipid hydroperoxide levels were determined as the peroxide values (POV). Lipids from o/w emulsions were extracted prior to POV analyses by mixing 10 g of sample with 100 mL of methanol:chloroform solution (2:1 v/v),

followed by centrifugation at 2500*g*. The chloroform fraction was recovered and evaporated completely under vacuum at 30 °C to obtain the extracted lipids. Bulk oil samples were submitted to POV analyses directly. The POV value was determined according to the AOCS method CB-8-90 (*44*). Briefly, approximately 0.5 g of oil was dissolved in 25 mL of acetic acid:isooctane solution (3:2 v/v). A saturated potassium iodide (KI) solution (0.25 mL) was added to the sample. Water (25 mL) was added exactly 1 min after the KI addition. The mixture was titrated with 0.01 N sodium thiosulphate solution using a starch solution as the color indicator solution.

Determination of Total Phenol Content by FC Assay. Oregano, parsley, and OMWW phenol content was determined by the FC assay (*45*) as described by Jimenez-Alvarez et al. (*46*). Total phenols were expressed as mg gallic acid equivalents (GAE) per g of original sample on a dry mass basis (mg GAE/g, db).

Preparation of Hydrophilic and Lipophilic Extracts. Hydrophilic and lipophilic extracts of oregano, parsley, and OMMW for measurement of antioxidant capacity in vitro were obtained using the procedure described elsewhere (*46*). Briefly, lipophilic extracts were obtained by mixing 1 g of ground sample (oregano, parsley, and OMWW) with 2 × 10 mL of hexane and centrifuging at 2500*g* for 5 min. Supernatants were combined and evaporated to dryness under nitrogen. The residue was reconstituted in 10 mL of acetone and filtered to obtain the lipophilic extract. The hydrophilic extract was obtained by mixing the residue from lipophilic extraction with 3 × 5 mL acetone/water/acetic acid (70:28:2 v/v/v) and centrifuging at 2500*g* for 5 min. Supernatants were combined in a 25 mL volumetric flask, and acetone/water/acetic acid solution (70:28:2 v/v/v) was added up to the volume prior to filtration.

ORAC Assay. The ORAC assay was performed as described by Huang et al. (*47*) with slight modification (*46*). Briefly, hydrophilic extracts and Trolox calibration solutions were prepared in phosphate buffer, pH 7 (disodium hydrogen phosphate/potassium dihydrogen phosphate, Merck KGaA., Switzerland). Sample (20 μL) and fluorescein solution (200 μL, 1.1 μM) were added to a 96-well microplate, and the mixture was incubated at 37 °C for 20 min, followed by addition of 75 μL of AAPH solution (63 mM). Fluorescence was monitored at 485 and 525 nm (excitation and emission wavelengths, respectively) at 1 min intervals for 90 min. The lipophilic ORAC assay was performed as described above, except that lipophilic extracts and Trolox were diluted in 7% (% w) RMCD in acetone:water (1:1 v/v). Results were expressed as μmol Trolox equivalents (TE) per g of original sample on a dry mass basis (μmol TE/g, db).

FRAP Assay. The FRAP assay was performed according to Benzie et al. (*48*) with minor modifications (*46*). Hydrophilic extracts and Trolox calibration solutions were prepared in milli-Q water. FRAP reagent was prepared by mixing 20 mL of acetate buffer (pH 3.6), 2 mL of TPTZ (10 mM, dissolved in 400 mM HCl), and 2 mL of iron(III) chloride (20 mM). Sample (25 μL) and FRAP reagent (250 μL) were added in a 96-well microplate and incubated at room temperature for 8 min, and the absorbance was measured at 593 nm. Lipophilic extracts were analyzed as described above, but the extracts were dissolved in 7% RMCD acetone:water (1:1 v/v) and Trolox calibration solutions in acetone:water (1:1 v/v). Results were expressed as μmol TE per g of original sample on a dry mass basis (μmol TE/g, db).

ICA Assay. The ICA assay was performed according to Carter (*49*) with slight modifications (*46*). EDTA calibration solutions and hydrophilic and lipophilic extracts were diluted in acetate buffer (pH 3.6). Sample (135 μL) and iron(II) chloride (15 μL, 1 mmol/L in methanol) were added to a 96-well microplate. The mixture was kept at room temperature for 20 min after which ferrozine (150 μL, 1 mmol/L in acetate buffer pH 3.6) was added. Absorbance was measured at 562 nm after 5 min of incubation at room temperature. Results were expressed as μmol EDTA equiv per g of original sample on dried basis (μmol EDTA equiv/g, db).

Particle Size Determination in o/w Emulsions. The size of the oil droplets in the o/w emulsions was determined by laser diffraction measurements using a Mastersizer (Malvern Instruments, United Kingdom). Samples were diluted in water, introduced into the measurement chamber, and irradiated with a helium/neon laser beam (632 nm, 5 mW). A Fourier-transform lens focused the light diffracted by the dispersed particles in the detector. Particle size results were reported as the volume mean diameter *D*[4,3], calculated by the method of Rawle (*50*):

$$D[4,3] = \sum d^4 / \sum d^3$$

where d is the diameter of a droplet (μm). Droplet size distribution percentiles at 10, 50, and 90% (*D*[v,0.1], *D*[v,0.5], and *D*[v,0.9], respectively) were reported as well. All of the parameters were calculated by the software (Mastersizer-S, Malvern Instruments).

Statistical Analyses. All experiments were performed in duplicate. Significance differences between samples were calculated by comparison of means using the Aspin−Welch test. Significance was declared at $p < 0.05$. Statistical analyses were performed using in-house software.

Table 1. Fatty Acid Composition of Tuna Oil

fatty acid name		amount (%)
myristic acid	C 14:0	3.1
myristoleic acid	C 14:1 n-5	0.1
pentadecanoic acid	C 15:0	0.7
palmitic acid	C 16:0	17.6
palmitoleic acid + isomers	C 16:1 n-7	5.5
margaric acid	C 17:0	1.7
heptadecenoic acid	C 17:1	1
stearic acid	C 18:0	4.8
oleic acid + isomers	C 18:1 n-9, n-7	18.3
linoleic acid	C 18:2 n-6	1.2
linoleic acid trans isomers	C 18:2 n-6	0.5
α-linolenic acid	C 18:3 n-3	0.5
γ-linolenic acid	C 18:3 n-6	0.1
stearidonic acid + cis isomers	C 18:4 n-3	0.8
arachidic acid	C 20:0	0.3
eicosenoic acid	C 20:1 n-9	1.8
eicosadienoic acid	C 20:2 n-6	0.3
eicosatrienoic acid	C 20:3 n-6	0.1
ARA	C 20:4 n-6	2.1
EPA	C 20:5 n-3	6.7
behenic acid	C 22:0	0.2
erucic acid	C 22:1 n-9	0.4
docosapentaenoic acid	C 22:5 n-6	1.2
docosapentaenoic acid (DPA)	C 22:5 n-3	1.5
DHA	C 22:6 n-3	22.9
lignoceric acid	C 24:0	0.1
nervonic acid	C 24:1 n-9	0.7
others		5.8

RESULTS AND DISCUSSION

Fatty Acid Compositions of Tuna Oil and MCT Oil. The major fatty acids in the tuna oil were docosahexaenoic acid (DHA, 22.9%), oleic acid (18.3%), palmitic acid (17.6%), and eicosapentaenoic acid (EPA, 6.7%) (**Table 1**). Arachidonic acid (ARA) was also present in tuna oil (2.1%). The total amounts of n-3 (e.g., DHA and EPA) and n-6 fatty acids (e.g., ARA) were 32.4 and 5.5%, respectively. MCT oil was used to dilute tuna oil samples. MCT oil contained mainly caprylic acid (C8:0, 54−64%) and capric acid (C10:0, 34−46%) with small amounts (below 2%) of caproic acid (C 6:0) and lauric acid (C 12:0) (information provided by the supplier). MCT fatty acids were saturated and as a consequence were stable to oxidation.

Markers of Volatile Compounds Formed by Oxidation. Volatile compounds were observed to form during the storage of both bulk oil and o/w emulsions (**Figure 1**), although the levels were higher in the later. In o/w emulsions, several volatiles were identified (hexanal, *t*,*t*-2,4-heptadienal, *t*-2-octenal, *t*,*t*-3,5-octadiene-2-one, and nonanal), whereas in bulk oil, only hexanal, *t*,*t*-2,4-heptadienal, and nonanal were detected. In both systems, hexanal was the main volatile formed during the storage. Hexanal, *t*-2-octenal, and nonanal are oxidation products of n-6

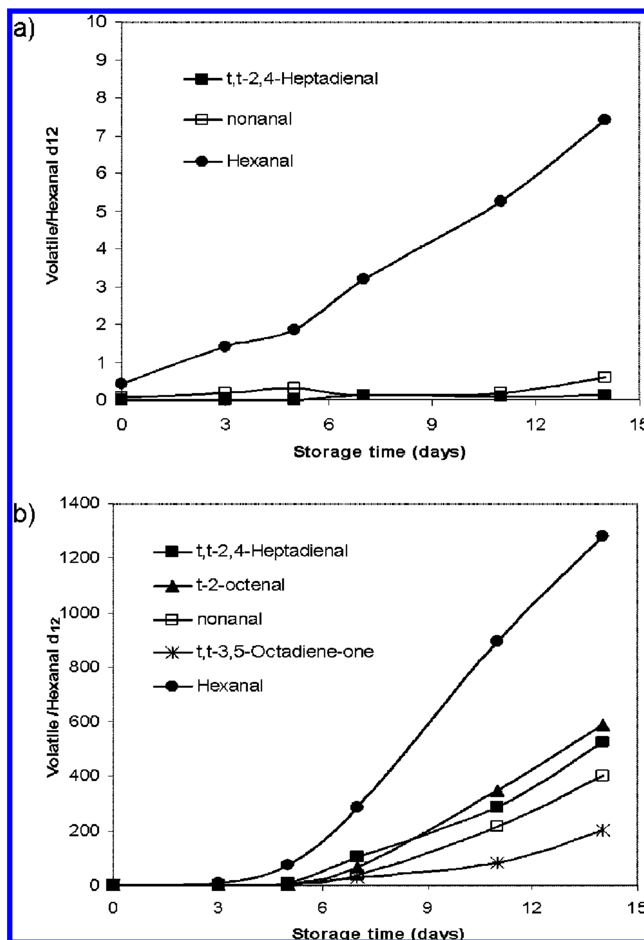

Figure 1. Formation profiles of several volatiles formed in (**a**) bulk oil and (**b**) o/w emulsion. In both physical systems, samples contained 5% tuna oil and were stored at 37 °C for 14 days. Peak areas were normalized against that of the internal standard (hexanal-d_{12}).

PUFAs fatty acids (*7, 51, 52*), whereas *t*,*t*-2,4-heptadienal and *t*,*t*-3,5-octadiene-2-one are oxidation products of n-3 PUFAs (*7, 22*). Hexanal has been widely used as a marker of volatile oxidation products of n-6 fatty acids in previous studies (*53*). *t*,*t*-2,4-Heptadienal has been correlated to fishy and rancid off-flavors developed in fish-oil-enriched mayonnaise (*54*) and has been used as a marker of oxidation of fish oils rich in n-3 fatty acids (*33, 37*). Accordingly, hexanal and *t*,*t*-2,4-heptadienal were chosen as markers of volatile oxidation compounds from n-6 and n-3 fatty acids, respectively.

Droplet Size in o/w Emulsions. Droplet size is an important parameter influencing the oxidative stability of o/w emulsions since it determines the surface area of the oil per unit of volume exposed to oxygen, pro-oxidants, and antioxidants (*9, 55*). It is well-established that lipid oxidation is accelerated by reactions that take place at the surface of droplets (*9*). To compare the effect of the addition of antioxidants on the stability of the emulsion system, the physical stability at the incubation temperature (37 °C) was determined by measuring the particle size at the beginning of the storage (**Table 2**) and at regular periods over 14 days. The volume mean diameter *D*[4,3]) and the percentiles of droplet size distribution (*D*[v,0.1], *D*[v,0.5], and *D*[v,0.9]) did not change significantly during the storage period (results not shown), indicating that the emulsions were physically stable during the experiments.

Oxidative Stability of Bulk Oil and o/w Emulsions. The oxidative stability of bulk oil and o/w emulsion, both containing 5% w/w tuna oil and Fe^{2+} (3.5 mg/kg), was evaluated by monitoring the levels of hydroperoxides and volatiles (hexanal and *t*,*t*-2,4-heptadienal) in the control samples (no added antioxidant) over 14 days of storage at 37 °C. An iron(II) chloride solution (acetate buffer, pH 3.6) was added to the samples to ensure similar levels of iron to those commonly found in foods such as mayonnaise, salad dressing, or olive oil (*22*). Iron is generally considered as a catalyst of lipid hydroperoxide decomposition to form volatile compounds (*7*). Fe^{2+} readily oxidizes to Fe^{3+} in aqueous solutions; thus, the iron added to the samples will have been present in the oxidized state, which is less catalytically active than the reduced one (*7*).

Table 2. Droplet Size Average Volume (*D*[4,3]) and Volume Median Values at 10, 50, and 90% (*D*[v,0.1], *D*[v,0.5], and *D*[v,0.9], Respectively) in o/w Emulsions Containing Different Antioxidants at the Beginning of the Storage Study[a]

	μm			
sample	*D*[v,0.1]	*D*[v,0.5]	*D*[v,0.9]	*D*[4,3]
control	0.39 ± 0.02	2.53 ± 0.64	16.70 ± 2.88	7.13 ± 1.83
EDTA	0.35 ± 0.01	1.62 ± 0.53	18.92 ± 6.06	7.37 ± 2.69
Trolox	0.37 ± 0.01	1.03 ± 0.17	16.90 ± 2.96	5.65 ± 0.93
oregano	0.41 ± 0.02	2.64 ± 0.60	14.22 ± 3.05	6.70 ± 1.08
OMWW	0.36 ± 0.01	1.54 ± 0.18	18.29 ± 0.70	6.72 ± 1.46
parsley	0.42 ± 0.06	2.54 ± 0.94	20.91 ± 7.83	7.75 ± 2.91

[a] No significant differences were found between the control and the antioxidant samples ($p < 0.05$).

In the bulk oil control, no hydroperoxides evolved until day 8 of storage, after which there was a sharp increase in the levels through to day 10 (**Figure 2a**). Hexanal and *t*,*t*-2,4-heptadienal levels increased during the storage period, rising rapidly after the fifth day of storage (**Figure 2b**,**c**). Both volatiles exhibit similar evolution profiles, although more hexanal was observed in relative terms.

The o/w emulsion was more susceptible to oxidation than the bulk oil, as shown by the considerably higher levels of hydroperoxides and volatiles that were formed (**Figure 3**). The higher oxidative susceptibility of o/w emulsions has been reported extensively, and it is attributed to the greater extent of interfacial interactions that are possible between the lipid substrate and the oxidants as a consequence of the greater surface area presented by the emulsion droplets (*9, 39, 55*). In o/w emulsion, hydroperoxides reached a level 10 times higher than in bulk oil at the end of the storage study. Hexanal and *t*,*t*-2,4-heptadienal levels reached 173 and 3674 times higher than in bulk oil, respectively, thus showing a larger relative increase in *t*,*t*-2,4-heptadienal as compared with hexanal. The total amounts of n-3 fatty acids and n-6 fatty acids in tuna oil (32.4 and 5.5%, respectively, **Table 1**) can only partially explain the differences in the relative increase of hexanal and *t*,*t*-2,4-heptadienal. The n-3 fatty acids in tuna oil were generally more unsaturated than the n-6 fatty acids (**Table 1**); as a consequence, n-3 fatty acids were more susceptible to oxidation, thus partially explaining the increased formation of *t*,*t*-2,4-heptadienal as compared to hexanal. Other factors (e.g., n-3 and n-6 hydroperoxides location) rather than fatty acid composition would contribute to the preferential formation of *t*,*t*-2,4-heptadienal. According to Jacobsen et al. (*55*), n-3 fatty acid hydroperoxides are more polar than n-6 hydroperoxides; thus, n-3 hydroperoxides may have a higher degree of partition in the aqueous phase of the o/w interface than n-6 hydroperoxides. In o/w emulsion, metal catalyst such as Fe^{3+} would be present in the aqueous phase and oriented in the o/w interface (*7, 55*). Therefore, an hypothesis is that n-3 hydroperoxides may be better suited for

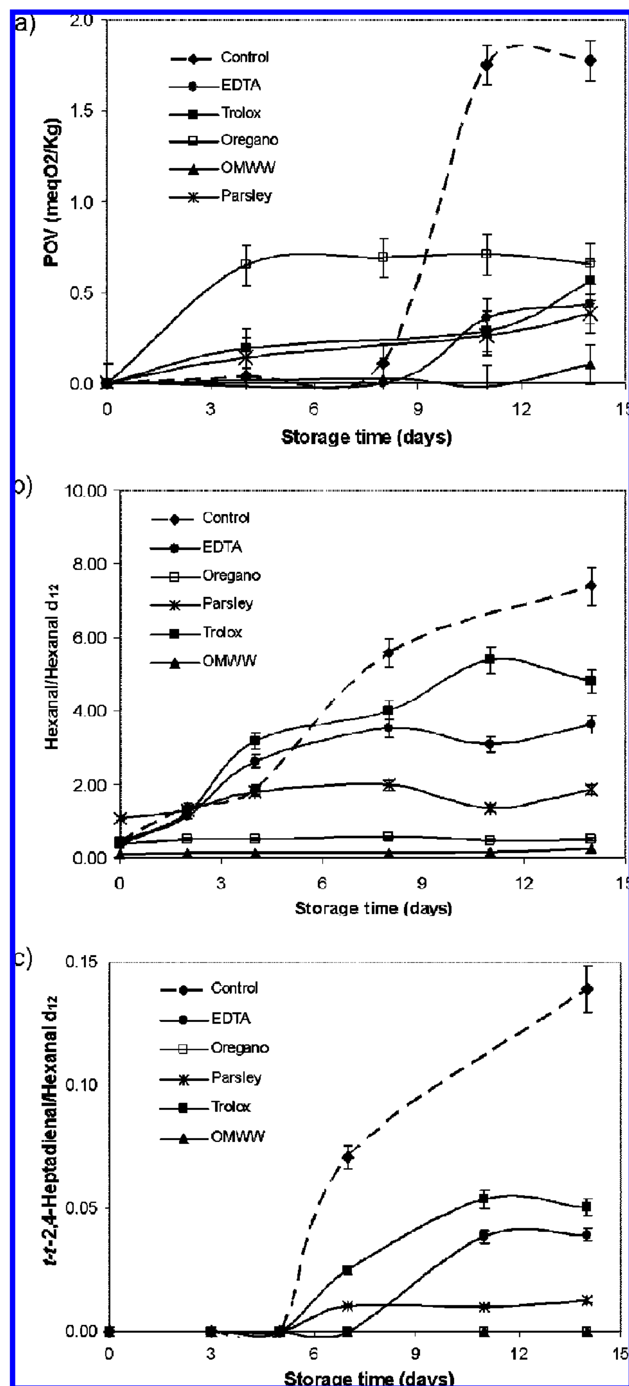

Figure 2. Evolution of oxidation compounds in bulk oil samples containing 5% tuna oil stored at 37 °C: (**a**) hydroperoxides, (**b**) hexanal, and (**c**) *t,t*-2,4-heptadienal. Each data point is the mean of duplicate analyses. Hexanal and *t,t*-2,4-heptadienal peak areas were normalized against that of the internal standard (hexanal-d_{12}). Error bars represent the standard deviation of the repeatability of the method.

reaction with metals at the o/w interface than n-6 hydroperoxides, and as a consequence, volatiles formed from n-3 hydroperoxides decomposition (e.g., *t,t*-2,4-heptadienal) would be expected to form rather than volatiles formed from the decomposition n-6 hydroperoxides (e.g., hexanal). Nevertheless, further research addressing the impact of lipid hydroperoxides location on the formation of volatile compounds is needed to confirm this hypothesis.

Antioxidant Activity in Bulk Oil and o/w Emulsions. To evaluate the impact of the physical properties of food on antioxidant activity, the efficacy of OMMW, oregano, parsley, EDTA, and Trolox to inhibit lipid oxidation was determined in both bulk oil and o/w emulsion enriched with fish oil.

EDTA. EDTA is widely recognized as an effective metal chelator (*55*). In bulk oils, EDTA (75 mg/kg) inhibited the formation of hydroperoxides, hexanal, and *t,t*-2,4-heptadienal by a 75.5, 51.0, and 71.7%, respectively (**Figure 2** and **Table 3**). EDTA showed better antioxidant properties in o/w emulsions (**Figure 3**), inhibiting the formation of hydroperoxides, hexanal, and *t,t*-2,4-heptadienal by a 76.8, 99.8, and 99.9%, respectively (**Table 3**). The greater efficacy of EDTA in o/w emulsion than in bulk oil indicates that metal-catalyzed oxidation plays a more important role in o/w emulsion than in bulk oil. Previous studies reported good antioxidant properties of EDTA in fish oil-enriched emulsions (*33–37*).

OMWW. OMWW (1% w/w) was the most effective antioxidant in bulk oil and o/w emulsions, greatly inhibiting the formation of hydroperoxides, hexanal, and *t,t*-2,4-heptadienal throughout the storage period (**Figures 2** and **3** and **Table 3**). OMWW inhibited hydroperoxide, hexanal, and *t,t*-2,4-heptadienal formation in bulk oil by 94.2, 96.5, and 100%, and similar levels of inhibition were observed in o/w emulsions (91.9, 99.6, and 99.9%).

The antioxidant properties of OMMW in food have been scarcely studied. De Leonardis et al. (*21*) observed that OMWW was very efficient inhibiting lipid oxidation in lard, and Medina et al. (*20*) reported the synergistic properties of OMWW in reinforcing the antioxidant properties of lactoferrin in liposomes and o/w emulsions of corn oil. To our knowledge, the antioxidant properties of OMWW in fish bulk oil and fish o/w emulsions have not been reported previously. The antioxidant properties of OMWW have been attributed to its high phenol content (*18*), phenols being recognized to have both radical scavenging and metal chelating properties (*56*). The total phenolic content of OMWW extracts was 87.6 mg GAE/g (**Table 4**). Several phenolic compounds, including hydroxytyrosol, tyrosol, elenolic acid, gluteolin 7-glycoside, quercetin, and cinnamic acid derivatives, have been previously identified in OMWW extracts (*16*). Hydroxytyrosol is the most abundant phenolic compound in OMWW, and its concentration has been reported previously to vary from 1.2 to 9.8% among different OMWW extracts (*15, 16*). The hydroxytyrosol content in the OMWW extract used in our study was 3.4% of dry solids (information provided by the supplier). Hydroxytyrosol has shown good antioxidant properties in foodstuffs rich in fish lipids such as frozen horse mackerel, bulk cod liver oil, and cod liver o/w emulsion (*57, 58*). Pazos et al. (*57*) reported the good antioxidant properties of hydroxytyrosol in both bulk fish oil and fish o/w emulsions. The antioxidant activity of hydroxytyrosol has been attributed to the 1,2-dihydroxyphenyl substructure, which acts both as a free radical scavenger and a metal chelator (*17*). From in vitro experiments, the OMWW extract showed good radical scavenging properties (1723 μmol TE/g, assessed by the ORAC assay) and a low iron chelating capacity (0.47 mg EDTA equiv/g, determined by the ICA assay; **Table 4**). Previous studies have shown the good in vitro metal chelating properties of hydroxytyrosol (*58*). To check the metal chelating properties of hydroxytyrosol, standard solutions (concentration range, 5−30 mg/L) were prepared in acetate buffer, pH 3.6, and submitted to ICA analyses. Hydroxytyrosol did not show chelating activity under the conditions used in the ICA assay. The lack of agreement with previous findings and the fact that, in our study, metal catalysts play a very important role in

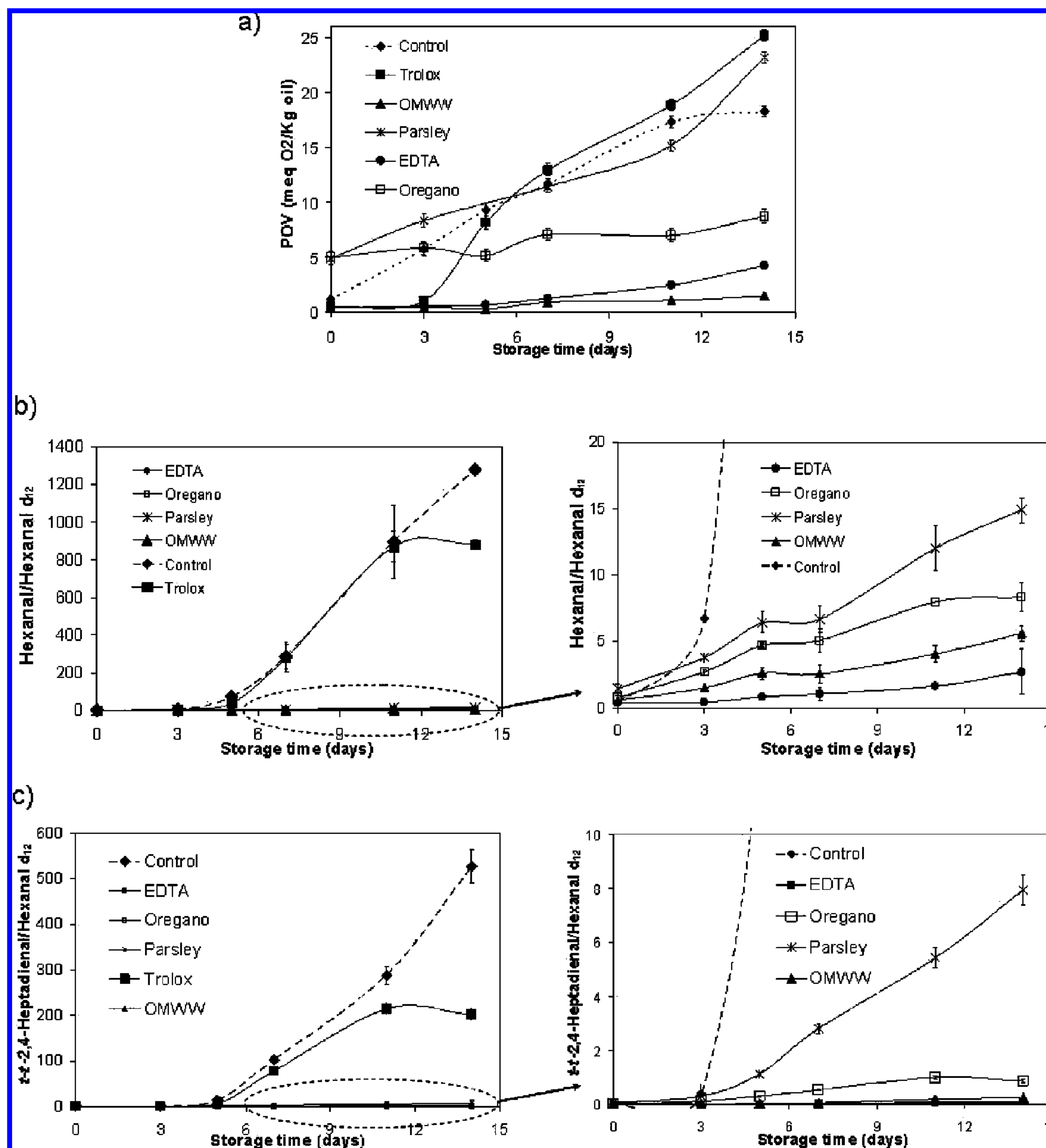

Figure 3. Evolution of oxidation compounds in o/w emulsion samples containing 5% tuna oil stored at 37 °C: (**a**) hydroperoxides formation, (**b**) hexanal evolution, and (**c**) *t*,*t*-2,4-heptadienal formation. Each data point is the mean of duplicate analyses. Hexanal and *t*,*t*-2,4-heptadienal peak areas were normalized against that of the internal standard (hexanal-d_{12}). Error bars represent the standard deviation of the repeatability of the method.

oxidation of food emulsions may indicate that ICA results may not provide reliable information of the metal chelating properties of OMWW.

Previous studies have shown a pro-oxidant effect of phenolic compounds in o/w emulsions, maybe due to its ability to reduce Fe^{3+} to Fe^{2+}, which is more catalytically active than ferric iron (*7, 12*). In our own study, no pro-oxidant effect was observed for OMWW, although it showed the ability to reduce Fe^{3+} to Fe^{2+} as determined by the FRAP assay (319 μmol/TE/g; **Table 4**). These results may suggest that the antioxidants present in OMMW were not able to reduce Fe^{3+} to Fe^{2+} under the conditions used in the tested foods.

Oregano. Oregano (1% w/w) greatly inhibited the formation of hexanal and *t*,*t*-2,4-heptadienal in bulk oil (93.1 and 100% inhibition, respectively) but was less effective in preventing hydroperoxide formation (63.0% inhibition; **Table 3**). Notably, oregano promoted the formation of hydroperoxides in the early stages of storage, and after 4 days of storage, hydroperoxide levels remained constant until the end of the storage study (**Figure 2a**). A low amount of hexanal was formed at the beginning of the storage period, and no further increase was observed during storage. *t*,*t*-2,4-Heptadienal was not detected in any of the measurements performed during storage (**Figure 2b**,**c**). As was observed for bulk oil, oregano showed a pro-oxidant effect on hydroperoxides at the early stages of the oxidation process in emulsions, with POV values at time zero of 5.0 mequiv O_2/kg oil (**Figure 3a**). At later stages, the rate of formation of hydroperoxides in the oregano sample was lower than in the control sample with inhibition of 52.2% at the end of storage, indicating its antioxidant effect. Oregano inhibited hexanal and *t*,*t*-2,4-heptadienal formation over the whole period of the storage of emulsions (**Figure 3b**,**c**), limiting the formation of hexanal and *t*,*t*-2,4-heptadienal at the end of the storage period by 99.3 and 99.8%, respectively (**Table 3**).

The oregano radical scavenging properties in vitro were determined as 1133 and 35 μmol TE/g by the hydrophilic (H-

Table 3. Percent Inhibition of the Formation of Oxidation Compounds at the End of the Storage Study (14 Days in Samples Containing 5% Tuna Oil and Antioxidants with Regard to the Control)[a]

		% inhibition		
physical system	sample	POV[b]	hexanal/ hexanal-d_{12}[c]	heptadienal/ hexanal-d_{12}[c]
bulk oil	control	NA	NA	NA
	EDTA	75.5	51.0	71.7
	oregano	63.0	93.1	100
	OMWW	94.2	96.5	100
	parsley	78.4	75.0	90.8
	Trolox	68.3	35.2	63.7
o/w emulsion	control	NA	NA	NA
	EDTA	76.8	99.8	99.9
	oregano	52.2	99.3	99.8
	OMWW	91.9	99.6	99.9
	parsley	−26.7	98.8	98.5
	Trolox	−37.8	31.2	61.6

[a] Legend: NA, not applicable. [b] POV expressed as mequiv O_2/kg oil. [c] Ratio of peak areas. Hexanal-d_{12} was used as an internal standard.

ORAC) and lipophilic (L-ORAC) assays, respectively (**Table 4**). ORAC results showed the presence of antioxidant compounds of different polarity in oregano extracts. The iron chelating properties were determined by the ICA assay as 1.36 mg EDTA equiv/g; thus, oregano (1%w/w) would chelate by stoichiometry the 74% of Fe^{3+} at the concentrations present in the sample. Nevertheless, FRAP results showed the ability of oregano to reduce Fe^{3+} to Fe^{2+} (257 μmol TE/g, **Table 4**), which may explain the pro-oxidant effect of oregano on hydroperoxides formation at early stages in bulk oil and o/w emulsions. Sorensen et al. (*12*) have reported a marked pro-oxidant effect of caffeic acid on the formation of hydroperoxides and volatile compounds in o/w emulsions at pH 3, maybe due to its ability to reduce ferric ion.

In both food systems, a dual antioxidant and pro-oxidant effect was observed for oregano on hydroperoxides formation at earlier and later stages of oxidation, respectively. The antioxidant behavior of oregano is attributed to the presence of phenolic compounds (e.g., caffeic acid and rosmaniric acid) with radical chain breaking and metal chelating properties (*22, 59*). On the other hand, under certain conditions, for example, high concentrations, phenolics produce unstable phenoxyl radicals that can promote hydroperoxide formation by reacting with the lipid substrate to form alkyl radicals, by regenerating peroxyl radicals or by reducing metals to a more catalytically active form (*60*). Oregano results showed the complex antioxidant/ pro-oxidant behavior of phenolic compounds in food.

Parsley. Parsley (1% w/w) inhibited the formation of oxidation products in bulk oil, although it was less effective as an antioxidant than OMMW and oregano. A pro-oxidant effect of parsley on hydroperoxides and hexanal formation was observed at early stages of the oxidation process (**Figure 2**). In later stages, the rates of formation of hydroperoxide and hexanal were low. Thus, at the end of the storage, parsley inhibited hydroperoxide and hexanal formation by 78.4 and 75.0%, respectively (**Table 3**). Parsley was a more efficient inhibitor of *t*,*t*-2,4-heptadienal formation (90.8% inhibition) than it was of hexanal formation. Parsley showed a pro-oxidant effect with respect to hydroperoxide formation in o/w emulsion throughout the storage study, with a POV at time zero of 4.9 mequiv O_2/kg oil and a hydroperoxide level 26.7% higher than the control sample at day 14 (**Table 3**). By contrast, parsley was very efficient at retarding the formation of hexanal and *t*,*t*-2,4-heptadienal with % inhibition of 98.8 and 98.5%, respectively. The behavior of parsley o/w emulsions indicates that the antioxidant compounds found in parsley are efficient at preventing volatiles formation while promoting an increase in the level of hydroperoxides.

The level of phenolic compounds in parsley, determined by FC assay, was 18.0 mg GAE/g. Parsley showed the best iron chelating properties (3.11 mg EDTA equiv/g; **Table 4**) and the lowest radical scavenging properties (656 μmol TE/g; **Table 4**) of the plant extracts tested. The amount of parsley present in the samples (1%) is equivalent to 3.11 mg EDTA, which would chelate by stoichiometry all of the Fe^{3+} present in the sample. Thus, the good iron chelating properties of parsley in vitro reported previously (*31, 61*) were confirmed in this study. Caffeic acid, the main phenolic compound present in parsley (*24*), has a 1,2-dihydroxyphenyl substructure, which is very efficient at complexing metals (*56*), although it can promote lipid oxidation by reducing Fe^{3+} to Fe^{2+} (*12*). FRAP results showed the iron reducing capacity of parsley (18 μmol TE/g; **Table 4**); thus, similarly to oregano, parsley phenolics may promote hydroperoxides formation by reducing Fe^{3+} to Fe^{2+}.

Trolox. In bulk oil (**Figure 2**), Trolox (75 mg/kg) slightly promoted hydroperoxide and hexanal formation during early stages of storage. During the later stages, it prevented the formation of oxidation compounds (**Table 3**). Trolox was less efficient in o/w emulsions than in bulk oils (**Figure 3**), in agreement with the polar paradox, which states that polar antioxidants are more efficient in o/w emulsions than in bulk oils. In emulsions, Trolox promoted hydroperoxide formation after day 7 of storage. Trolox inhibited hexanal formation (31% inhibition) during the later stages of storage (**Table 3**), whereas it was more efficient at preventing *t*,*t*-2,4-heptadienal formation (61.6% inhibition at day 14). The greater efficiency of Trolox at preventing *t*,*t*-2,4-heptadienal formation than hexanal formation in bulk oil and o/w emulsions may be explained by the higher polarity of n-3 hydroperoxides with regard to n-6 hydroperoxides (*55*). The degree of partition of n-3 hydroperoxides into the aqueous phase, where Trolox would be expected to be located, may be higher than the one of n-6 hydroperoxides; thus, Trolox would be more effective preventing n-3 hydroperoxides decomposition.

Correlation of in Vitro Experiments and Antioxidant Efficacy in Food. The antioxidant activities of OMWW, oregano, and parsley both in bulk oil (**Figure 2**) and in o/w emulsions (**Figure 3**) correlated with the total phenol contents and radical scavenging capacity in vitro (**Table 4**). Results from

Table 4. In Vitro Antioxidant Capacity of Parsley, Oregano, and Olive Mill Waste Waters (OMWW) Analyzed by the ORAC, FRAP, and ICA Methods[a]

	ORAC (μmol TE/g)			FRAP (μmol TE/g)			ICA (mg EDTA/g)			
sample	L-ORAC	H-ORAC	total ORAC	L-FRAP	H-FRAP	total FRAP	H-ICA	L-ICA	total ICA	total phenols (mg GAE/g)
parsley ($n = 2$)	2.85 ± 0.1	653 ± 27	656 ± 27	0.60 ± 0.02	17.2 ± 0.8	17.8 ± 0.8	3.11 ± 0.24	ND	3.11 ± 0.24	18.0 ± 0.8
oregano ($n = 12$)	35 ± 5	1133 ± 104	1168 ± 105	2.03 ± 0.25	254.6 ± 11.6	256.7 ± 11.8	1.36 ± 0.11	ND	1.36 ± 0.11	60.7 ± 2.0
OMWW ($n = 2$)	ND	1723 ± 15	1723 ± 15	ND	319.4 ± 8.6	319.4 ± 8.6	0.47 ± 0.09	ND	0.47 ± 0.09	87.6 ± 2.5

[a] Total phenols were quantified by the Folin−Ciocalteu assay. Results are expressed as the mean ± one standard deviation. Legend: ND, not detectable; *n*, number of replicates; L, lipophilic; and H, hydrophilic.

the iron(II) chelating assay did not show a clear correlation with the antioxidant capacity in bulk oil and o/w emulsions. Although in vitro methods provide useful information about the mechanisms of action of antioxidants, a significant limitation is that their use does not take into account important factors that could affect antioxidant activity in food, such as the colloidal properties of the substrates or the physical location of the antioxidant (*62*). To obtain reliable results on the capacity of a substance or preparation to act as an antioxidant and to delay lipid oxidation, evaluation has to be carried out on the food matrix itself, where all of the factors affecting lipid oxidation and antioxidant behavior are taken into account.

In summary, the order of effectiveness of natural antioxidants and Trolox was similar in both bulk oil and o/w emulsions. Notably, however, EDTA (75 mg/kg) exhibited a significant difference, being the most effective antioxidant in o/w emulsions, whereas in bulk oil it, performed similarly to oregano, parsley, and Trolox. The antioxidant properties of OMWW, oregano, and parsley are probably due to the presence of a mixture of compounds that possess different physical properties and act as antioxidants via different mechanisms. The effectiveness of OMWW, parsley, and oregano as antioxidants relates to their total phenol content and ORAC but not to the iron(II) chelating activity in vitro. Nevertheless, further investigations must address the identification of the active compounds in all three cases.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidino propane) dihydrochloride; ARA, arachidonic acid; DHA, docohexaenoic acid; EPA, eicosapentaenoic acid; EDTA, ethylenediaminetetraacetic acid; FC, Folin−Ciocalteu; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; ICA, iron(II) chelating assay; LC-PUFA, long-chain polyunsaturated fatty acids; MCT, medium-chain triglycerides; o/w, oil-in-water; OMWW, olive mill waste waters; ORAC, oxygen radical absorbance capacity; POV, peroxide value; RMCD, randomly methylated-β-cyclodextrin; HS-SPME-GC/MS, head space solid-phase microextraction−gas chromatography−mass spectrometry; TE, Trolox equivalents.

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