“Oxidative stability of O/W and W/O/W emulsions: effect of lipid composition and antioxidant polarity”

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ABSTRACT

The effect of storage temperature (65°C, 48 hours) on the oxidative stability of a food-grade water-in-oil-in-water (W/O/W) emulsion was studied by comparison with an oil-in-water (O/W) emulsion. The emulsions were prepared with linseed oil or olive oil, and in each case, two antioxidants were evaluated, an aqueous *Melissa* lyophilized extract and BHA. Emulsions were characterized using brightfield light microscopy and the oxidation was monitored by measuring the lipid hydroperoxides, thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD) and trienes (CT), alpha-tocopherol and Lipophilic Oxygen Radical Absorbance Capacity (L-ORACFL) Assay.

A great stability of olive oil emulsions was observed, without noticing differences between antioxidants or type of emulsion. This behaviour was not observed in linseed oil emulsions. In this case the lipophilic antioxidant (BHA) seemed to be more efficient delaying the lipid oxidation in W/O/W emulsions than the water *Melissa* extract while the opposite occurs in the O/W emulsion. The type of antioxidant is a key factor in controlling oxidation in W/O/W and O/W emulsions which are prepared with highly polyunsaturated oils, but not in the case of highly monounsaturated ones.

Key words: linseed oil, olive oil, antioxidant, multiple emulsion, lipid oxidation, lemon balm

1. INTRODUCTION

The use of vegetable oils as functional ingredients in food lipids emulsions might be complex from a technological point of view due to the high oxidation susceptibility of these unsaturated oils (Jacobsen, Timm & Meyer, 2001; Taherian, Britten, Sabik, & Fustier, 2011). It is known that oxidation may lead to production of rancid odours, unpleasant flavours and even compromise the safety of foods because of the formation of harmful compounds. Lipid oxidation can occur rapidly in emulsions due to their large surface area that facilitates interactions between the lipids and the water-soluble prooxidants. There are many factors that can potentially influence the physical and oxidative stability of emulsions: fatty acid composition, pH and ionic composition, type and concentration of antioxidants and prooxidants, emulsion droplet and interfacial properties, lipid droplet characteristics, concentration and physical state (McClements & Decker, 2000; Raikos, 2010; Naji, & Karazhiyan, 2012; Xu, Wang, Jiang, Yuan, & Gao, 2012).

Several studies have been carried out to elucidate lipid oxidation mechanisms in oils, providing important insights into the factors that influence lipid oxidation and strategies to control it (Waraho, McClements & Decker, 2011). The most common method to increase the oxidative stability of emulsion systems is probably the use of antioxidants (Gutteridge & Halliwell, 2010). Antioxidants are potentially able to be located in a number of different physical environments within a conventional oil-in-water emulsion (O/W) or in a multiple water-in-oil-in-water emulsion (W/O/W). Hydrophilic components can be incorporated by dispersing them in the external water phase in an O/W emulsion or into the internal aqueous phase in a W/O/W emulsion. Lipophilic components can be incorporated into the oil droplets by dispersing them in the oil phase (McClements, 2010). Porter (1993) first described the "antioxidant paradox" as a phenomenon where hydrophilic antioxidants were more effective than lipophilic antioxidants in bulk oils while lipophilic antioxidants were more effective in emulsified ones. This observation was attributed to the ability of non-polar antioxidants to

concentrate in the lipid phase of emulsions, whereas polar antioxidant partitioned in both the lipid and water phases (Laguerre et al., 2010; Shahidi & Zhong, 2011; Sorensen et al., 2011). According to this hypothesis, the efficacy of an antioxidant contained by the emulsion is affected by its polarity and its location in the different phases (Sorensen et al., 2011). Although the theory has been generally accepted, new evidence from more comprehensive assessments has more recently emerged and disagrees with the polar paradox, hence requiring a revaluation to this hypothesis (Shahidi et al., 2011). Several recent studies have shown that not all antioxidants behave according to the polar paradox hypothesis, indicating that antioxidant activity in complex systems is more complicated than previously assumed (Laguerre, Lecomte, Figueroa-Espinoza, & Barea, 2009).

Synthetic phenolic antioxidants, such as Butylated Hydroxyanisole (BHA), have been used as effective additives to control lipid oxidation in high fat content foods. However, an increasing concern about their safety has resulted in a preferential research on natural antioxidants (Lee & Kunz, 2005). A number of studies deal with the use of natural antioxidant extracts such as grape seed, rosemary, blackseeds, green tea, among others, in order to protect emulsions and other foods from oxidation (Samotyja & Malecka, 2007; Ramful et al., 2011; Gibis & Weiss, 2012). *Melissa officinalis* aqueous extracts have shown antioxidant activity tested in O/W emulsion made with linseed and algae oil (García-Iñiguez de Ciriano et al., 2010) but the hypothetical advantages to minimize lipid oxidation using W/O/W *versus* O/W emulsions systems has not previously been reported. Moreover, it has been said that the use of W/O/W systems for food applications is further limited by lack of suitable food-grade emulsifiers and stabilizers for the inner and outer emulsions (Sapei, Ali Naqvi, & Rousseau, 2012). According to these authors, shelf-life stability of these emulsions at high temperatures must be ascertained prior to their successful usage in foods.

The aim of this paper was to evaluate the oxidative stability of W/O/W emulsions compared to O/W ones, under the use of a lyophilized water extract of *Melissa officinalis* and BHA as

natural and synthetic antioxidants respectively. The type of antioxidant used and the phase in which they are placed might be relevant factors that can affect their effectiveness in different emulsion systems. Furthermore, taking into account the different oxidation susceptibility of oils depending on their unsaturation degree, two different oils were evaluated (linseed and olive oil).

2. MATERIALS AND METHODS

2.1. Materials

The oils used in this study were Extra Virgin Olive Oil (Aceites del Sur, Coosur S.A., Jaén, Spain) and Linseed Oil (Biolasi Productos Naturales S.L., Guipúzcoa, Spain), which were obtained using the cold-pressed method. The lipid profile of both oils was analyzed by gas chromatography (Table 1). 2-thiobarbituric acid, α-tocopherol acetate 98 %, α-tocopherol 97 %, tetraethoxypropane, fatty acid methyl esters, gallic acid monohydrated >98%, fluorescein sodium salt and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid 97%) were purchased from Sigma-Aldrich Chemical (Steinheim, Germany). Boron trifluoride/methanol, Folin-Ciocalteu's phenol reagents, butylated hydroxyanysole (BHA) and butylated hydroxytoluene (BHT) were obtained from Merck (Barcelona, Spain). Potassium hydroxide, hexane, cyclohexanone, methanol, hydrochloric acid, trichloroacetic acid, ammonium sulphate, monopotassium phosphate, dipotassium phosphate, Polysorbate 80, sodium carbonate and acetone were supplied by Panreac (Barcelona, Spain). Ethanol was purchased from Oppac (Navarra, Spain) and HPLC grade methanol from Scharlab (Barcelona, Spain). *k*-Carrageenan and soya protein were from Cargill (San Sebastian, Spain) and ANVISA (Madrid, Spain) respectively. AAPH (2,2'- Azobis(2-methylpropionamidine) dihydrochloride 98%) was from Across Organic (New Jersey, USA). RMCD (randomly methylated *β*-cyclodextrin) was purchased from Ciclolab R&D Dltd. (Budapest, Hungary).

Melissa officinalis dried leaves were purchased from Plantaron S.L. (Barcelona, Spain) and the aqueous *Melissa* extract was obtained following the method described by García-Íñiguez

de Ciriano et al. (2010). Chemical characterization of this extract was done, giving rise to a DPPH value of 552.13 mg Trolox Equivalent/g lyophilized extract and to an ORAC value of 1728 mg Trolox Equivalent/g lyophilized. A more detailed characterization of water and hydroalcoholic lyophilized extracts of *Melissa* was presented in Encalada et al. (2011).

2.2. Emulsion preparation

8 types of emulsions were prepared in the 2x2x2 experiment that was designed in this paper. Linseed oil (L) and olive oil (O) were used to prepare two types of emulsions, an oil-in-water (O/W) and a water-in-oil-in-water (W/O/W). Two antioxidants, aqueous *Melissa* extract (Mel) and butylated hydroxyanysole (BHA), were tested for each one of the emulsions.
The corresponding codes for the 8 types of emulsions were:
L:Mel:O/W, L:Mel:W/O/W, L:BHA:O/W, L:BHA:W/O/W, O:Mel:O/W, O:Mel:W/O/W, O:BHA:O/W and O:BHA:W/O/W. Amounts of each ingredient shown in the following lines are referred to 100g of emulsion.
The O/W emulsions were prepared by mixing 42.1g of water and 5.3g of isolated soya protein (2 min). Afterwards 52.63g of oil were slowly added and gently stirred in order to obtain a stable emulsion (3 min, 16.000 rpm, Ultra-Turrax® T25basic, IKA®WERKE, Germany). Two different batches were prepared depending on the antioxidant: a BHA batch in which 20mg of the synthetic antioxidant was added into the oily fraction and another one in which 47.7mg of *Melissa* was added into the water. The concentration of *Melissa* is equivalent to tenfold the antioxidant activity of BHA measured by the DPPH method. This amount was successfully used in a previous experiment in which the stability of the O/W emulsions that were rich in unsaturated fatty acids was tested at room temperature. (García-Iñiguez de Ciriano et al., 2010).
The W/O/W emulsions were carried out by a two step protocol that consisted on the preparation of a W/O simple emulsion and a further addition of this emulsion to a second aqueous phase. The simple W/O emulsion was prepared by adding the aqueous phase

(14.04g, that included 1% carrageenan and the antioxidants: 34.99mg of Melissa or 14.67mg BHA) to the oil phase (38.61g, that contained the hydrophobic surfactant: 0.03% Polysorbate 80). Both phases were previously heated separately to 70°C and then mixed. After the homogenization process (2 min, 16.000 rpm, Ultra-Turrax® T25basic) the emulsions were cooled down to room temperature, allowing the k-carrageenan to polymerize. In the second step, 42.1g of water and 5.3g of isolated soya protein were mixed (2 min). Afterwards 52.6g of the previously W/O emulsion were incorporated. The W/O emulsion was slowly added and gently stirred in order to obtain a stable W/O/W emulsion (3 min, 16.000 rpm, Ultra-Turrax® T25basic).

In W/O/W emulsions the oil/antioxidant ratio was the same as in the O/W emulsion.

2.3. Microscopic image analysis

The patterns of the emulsions were checked by a Nikon E-800 (Kawasaki, Japan) brightfield light microscopy with 40x magnification. The emulsions were observed after 24 hours of refrigeration at 4°C. 4.0 µl of the emulsion was trickled through microsyringe over a drop of water (3 µl) previously deposited on a slide (76x26 mm) and covered after the extension with a coverslip (24x32 mm). The images were monitored and captured by digital Nikon DXM-1200. The analyses were performed in triplicate, and the particle sizes were determined from the images using the application image analySIS^D 5.0 Olympus BioSystems GmbH (Soft Imaging System GmbH). The distribution of particles sizes was calculated for diameters lower than 4 µm, between 4-7 µm, 7-10 µm and higher than 10 µm.

2.4. Accelerated oxidation study

To study the susceptibility of the emulsions to oxidation, the samples were subjected to an accelerated oxidation test under standardized conditions. Emulsions (60 g) were placed in 125 mL flasks which were kept sealed during 48 h under Schaal oven test conditions (65 °C). Samples were taken every 24 h for analysis. It has been previously established that 1 day of

storage under this condition is equivalent to 1 month of storage at room temperature (Abou-Gharbia, Shehata, Youssef & Shahidi, 1996). The experiment was performed in duplicate.

2.5. Oil extraction

The method stated by Folch, Lees and Stanley (1957) was used for the extraction of the lipid fraction from the emulsions heated during 48 h. This lipid fraction was used for the analysis of the lipid profile, peroxide value, α-tocopherol and L-ORAC.

2.6. Lipid fraction analysis

Fatty acid profile was obtained by gas chromatography FID detection, previous preparation of the fatty acid methyl esters derivatives. Boron trifluoride/methanol was used for the preparation of fatty acid methyl esters (AOAC, 2002). A Perkin-Elmer Clarus 500 gas chromatograph (Madrid, Spain), equipped with a split-splitless injector, automatic autosampler, and coupled to a computerized system for data adquisition (TotalChrom, version 6.2.1) was used. It was fitted with a capillary column SP^{TM}-2560 (100 m×0.25 mm×0.2 µm; Sigma-Aldrich). The temperature of the injection port was 250 ºC and detector was 260 °C, the oven temperature was programmed to increase from 170 to 200 °C at a rate of 10.0 °C/min and then at rate of 4.0 ºC/min to 220 ºC. The carrier gas was hydrogen, 30.0 psi. The sample size was 0.5 µl and the split ratio was 120. The quantification of individual fatty acids used heptadecanoic acid methyl ester as internal standard. The identification of the fatty acids was done by comparison of their retention times with those of pure fatty acid methyl esters.

2.7. Oxidative analysis

2.7.1. Peroxide value

Peroxide value (PV) was analyzed in the extracted fat according to the AOAC Official Method (AOAC, 2000). Results were expressed in meq O_2/kg fat.

2.7.2. Conjugated dienes and trienes

A modification of the method described by Frankel, Huang, Aeschbach and Prior (1996) was used for the determination of conjugated dienes (CD) hydroperoxides. The lipid extract of the emulsion sample (0.1 g) was dissolved in 5.0 ml of methanol and vortexed for 30 seconds. The absorbance was measured at 234 nm using a Lambda 5 UV–Vis Spectrophotometer (Perkin Elmer, Paris, France). A filtration through 0.20 µm filter (Syringe-driven Filter Unit, Millex®) was applied just before the measurement to remove the protein fraction from the oil extracted sample and thereby diminish its spectrum interference in this region (Dimakou, Kiokias, Tsaprouni, & Oreopoulou, 2007). The trienes were measured according to the method previously mentioned. The absorbance was measured at 280 nm. Results were measured as the increase in absorbance value.

2.7.3. TBARS (Thiobarbituric acid value)

TBARS values were determined on the emulsions according to the method described by Masqsood and Benjakul (2010) with slight modifications. Briefly, the TBARS reagent was prepared by mixing 15% w/v trichloroacetic acid, 0.0375% w/v 2-thiobarbituric acid in 0.25N hydrochloric acid. The emulsion sample (0.5 g), 0.5ml of distillate water, 20µL of BHT (1%) and the TBARS reagent (2 mL) were vortexed in a centrifuge tube for 30 sec, placed in a boiling water bath for exactly 15 min and then cooled down in an ice bath to room temperature. Cyclohexanone (4 mL) and ammonium sulphate (1 mL, 4M) were added to the mixture and were vortexed for 30 sec. The mixture was centrifuged at room temperature at 4000 rpm for 10 minutes. The supernatant was collected and the absorbance was measured at 532 nm. A calibration curve TEP (tetraethoxypropane) was done for quantification purposes, using the same procedure as with the sample. Results were expressed in mg of malondialdehyde (MDA) equivalents/kg oil.

2.8. Antioxidant capacity

2.8.1 α-Tocopherol analysis

The α-tocopherol (α-TOH) content was determined by HPLC-UV analysis according to the method described by Berasategi, Barriuso, Ansorena and Astiasarán (2012). 0.1 g of the lipid extract of the emulsion sample and 0.1 mL of internal standard (α-tocopherol acetate 10 mg/mL solved in methanol) were filled up to 10 mL with previously warmed (30 ºC) supergradient HPLC grade methanol. Dilution was vortexed for 30 sec and filtered with 0.20 µm filter (Syringe-driven Filter Unit, Millex®). UV spectra were recorded on a Perkin Elmer UV-Vis Lambda 200 Series equipped with a photodiode array detector Series 200 PDA, using an analytical precolumn (3.8 mm x 8 mm with 4 mm x 3 mm of C18 cartridges, Phenomenex, California, USA) and a Supercosil LC18 column (25 mm x 4.6 mm, 5 µm particle size; Perkin Elmer Brownlee columns, Massachusetts, USA). A total of 10 µL of the sample was injected into the HPLC system and a isocratic elution with methanol/water (97:3) at 1.5 ml/min flow was performed. The UV acquisition was recorded at 292 nm for a 12 min run. Identification of α-tocopherol was done using the retention time of the pure standard compound (RT = 4.5 min) (Vitamin E 97 %) and its characteristic UV spectra. The quantification was performed using a calibration curve previously plotted with tocopherol acetate (RT = 7.5 min) (Vitamin E acetate 98 %).

2.8.2. Determination of Total Phenolic content (TPC)

TPC was determined spectrophotometrically following the Folin-Ciocalteu colorimetric method described by Herchi et al. (2011) with slight modifications. A one-gram oil sample was weighed, dissolved in 10 ml hexane and transferred to a separatory funnel. Then, 20 ml of a methanol-water mixture (80:10 v/v) were added. After 3 min of shaking, the lower methanol-water layer was removed. The extraction was repeated twice and the methanol-water phases were combined. The methanol-water extract was driven to dryness in a rotary evaporator under vacuum at 40°C. The dry residue was then dissolved in 1 mL of methanol. The composition of the reaction mixture was of 0.1 ml of suitable diluted sample, 7.9 ml of distilled water, 0.5 ml of Folin-Ciocalteu´s reagent, and 1.5 ml of 20 % sodium carbonate

anhydrous solution (added 2 minutes after the Folin-Ciocalteu´s reagent). After the initial mixing, the tubes stood at room temperature for 2 hours in the dark. The optical density of the blue-colored resulting solution was measured at 765 nm using a Lambda 5-UV-VIS spectrophotometer. The total phenolic content was expressed as mg gallic acid/kg oil, using the corresponding calibration curve. Absorbance measurements were made in duplicate for each diluted solution.

2.8.3. Lipophilic Oxygen Radical Absorbance Capacity (L-ORAC$_{FL}$) Assay.

L-ORAC$_{FL}$ assays were performed similarly to those described by Prior et al. (2003). The lipid extract of the emulsion sample (3-5 mg) was dissolved in 400 µL acetone and then diluted with 4.6 mL of a 7% RMCD solution (1:1, acetone/water, v/v). Samples were shaken at room temperature on an orbital shaker operating at 180 rpm for 1 hour before use. A 0.5 M stock solution of Trolox was prepared in 10mM phosphate buffer, and divided into 1 mL aliquots, which were stored at -20°C until use. A new set stock Trolox vials were taken from the freezer daily for the preparation of the samples on order to accomplish the standard-addition procedure (0, 5, 12, 20 µM) and the quality controls (12.5 and 50 µM). The 7% RMCD solution was used as blank, to dissolve the Trolox quality controls and to prepare the samples. To conduct the L-ORAC$_{FL}$ assay, 40 µL of the lipophilic solution and 120 µL of the fluorescein solution (132.5 nM) were added to the 96 well black plate. The microplate was equilibrated (5 min, 37°C) then the reaction was initiated by the addition of AAPH (40 µL, 150mM); readings were obtained immediately, in a FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany). The antioxidant capacity was expressed as mols Trolox equivalent/100 g oil.

2.9. Data analysis

Mean and standard deviation of all replicates were calculated. For each parameter, one factor ANOVA with Tuckey-b post hoc multiple comparisons was used in order to evaluate the significant differences through time. The correlation between peroxide value and conjugated

dienes in linseed oil were evaluated by Pearson's correlation test. Within each type of oil and type of emulsion the differences between antioxidants were evaluated by Student t-test.
The statistical analysis of data was done using the SPSS 15.0 program (SPSS, INC., Chicago, IL, USA). Significance level of $p \leq 0.05$ was used for all evaluations.

3. RESULTS

The microscopic images of the two types of emulsions confirmed the adequate structure of both the simple (O/W) and the double emulsion (W/O/W) (figure 1). In the case of the double emulsion, a combination of single inner droplets and several inner droplets was found, as described by Florence and Whitehill, (1981) for this type of emulsions. The mean particle diameter in the O/W emulsions ranged from 2.92-12.71 μm, while the diameter of W/O/W emulsions ranged from 2.76-13.06 μm. The size distribution of the oil droplets was, in the O/W emulsions, 11.79% (< 4 μm), 34.96% (4-7 μm), 29.27% (7-10 μm) and 23.98% (> 10 μm). In the W/O/W emulsions the size distribution was 21.79% (< 4 μm), 42.31% (4-7 μm), 8.97% (7-10 μm) and 26.92% (> 10 μm). With this distribution the physical stability was maintained along the experiment and no syneresis was noticed during the whole accelerated oxidation test.
The mean value for total fat content of the emulsions was 52.59g/100g and 38.61g/100g for O/W and W/O/W samples emulsions, respectively. The fatty acid profile of the emulsions after 48h at 65°C was compared to the profile of the oils in order to elucidate potential undergoing changes during the accelerated oxidation study. The α-linolenic and linoleic acid were the predominant PUFA of the total fatty acid for the linseed oil, accounting for 52.94 and 14.84 g/100 g oil respectively. On the other hand, oleic acid (78.27 g/100g oil), as expected was the most prevalent MUFA in olive oil. No significant modifications were found in the two representative fatty acids of both emulsion oils after the accelerated oxidation study compared to those in the unheated oil (data not shown).

Primary oxidation products were measured by PV (figure 2). The initial oxidation status of the 8 types of emulsions showed significant higher PV for olive oil containing samples compared to the linseed oil ones, i.e. 14.28 meq O_2/kg oil and 2.86 meq O_2/kg oil for BHA:O/W emulsions with olive oil and linseed oil, respectively. PV values in olive oil emulsions (including O/W and W/O/W) kept their values between 11.7–17.6 meq O_2/kg oil without showing statistical differences at the end of the treatment between the two antioxidants ($p<0.05$). In linseed oil emulsions, gradual increases in PV were observed, with different behaviour depending on the antioxidant and the type of emulsion. In O/W emulsions, aqueous *Melissa* extract seemed to be more effective than BHA ($p < 0.05$) delaying the increase in PV. Only a slight increase was noticed after 48h of treatment (up to 6.32 meq O_2/kg oil), while emulsions with BHA showed 14.14 meq O_2/kg oil. Regarding W/O/W emulsions, *Melissa* extract showed significantly higher values at 48h (10.61 meq O_2/kg oil) than those with BHA (8.57 meq O_2/kg oil) ($p<0.05$) for linseed oil emulsions.
The analysis of primary oxidation products was completed with the conjugated dienes and trienes measurement (table 2). Results showed that CD value tended to increase with storage time in all cases ($p < 0.05$). Moreover, W/O/W emulsions showed lower CD increments during the storage than their O/W counterparts, whatever the antioxidant was. CD behaved similarly in linseed oil emulsions (with increments during storage of 1.05-1.11 for O/W and 0.76-0.79 for W/O/W ones) and olive oil emulsions (increments O/W: 1.07-1.11; W/O/W: 0.48-0.72). Increases in CT were also observed in all emulsions during storage. BHA emulsions showed higher increments in the CT values than the Melissa emulsions in linseed oil samples in both types of systems (O/W: 0.14 and 0.06; W/O/W: 0.14 0 and 0.09).
Secondary oxidation products were monitored by the TBARS test (figure 3). As expected, very different behaviour of TBARS depending on the type of oil and emulsion was detected. In olive oil emulsions, TBARS value was stable and low in O/W emulsions, with no significant differences between *Melissa* and BHA throughout the accelerated oxidation study.

In W/O/W emulsions, a trend toward higher values compared to O/W emulsions was noticed, without differences between antioxidants. Additionally, in linseed oil emulsions TBARS values gradually increased during the storage period, being the increase faster in the first 24 hours in O/W emulsions than in W/O/W emulsions. However, after 48 hours of storage, Mel:O/W stabilized their TBA value, while the other three types of emulsions continued increasing. A maximum of 19.79 ± 1.22 mg/kg was found for BHA:O/W, which was 1.74-fold higher than Mel:O/W, 1.35-fold higher than BHA:W/O/W and no significant differences in Mel:W/O/W.

The initial antioxidant capacity measured by L-ORAC (figure 4) showed higher values for linseed oil emulsions containing samples (8315.4 µmol Trolox equivalent/100g oil on average) than those for olive oil (1978.9 µmol Trolox equivalent/100g oil). Storage significantly decreased the antioxidant capacity of all the emulsions particularly in the case of linseed oil. Data indicated that the antioxidant capacity of different emulsions differs depending on the type of antioxidant. Moreover, in linseed oil emulsions, *Melissa* extract showed a faster decrease in the antioxidant capacity than BHA during storage, in contrast to olive oil emulsions. In W/O/W emulsions, the decrease in the antioxidant capacity was slower than what was observed in O/W emulsions.

Results for α-TOH are shown in figure 5. Linseed oil emulsions were naturally higher in α-TOH (64.93 mg α-tocopherol/100g oil on average) compared to olive oil ones (31.52 mg α-tocopherol/100g oil on average). Once again, in linseed oil, a gradual decrease was observed in all the emulsions with different behaviour depending on the antioxidant used and the type of emulsion. Higher α-TOH content was detected in *Melissa* extract containing samples compared to BHA. No changes or a slight trend to decrease was noticed during time. In olive oil, the two antioxidants behaved similarly during time, maintaining constant values in the W/O/W emulsions and slightly increasing in the O/W emulsions.

4. DISCUSSION

W/O/W emulsions contain much lower amount of fat than O/W ones (14g less of fat per 100g emulsion), which is an aspect to be taken into account when they are used as ingredients for functional foods. Although there is widespread recognition of the perceived value of W/O/W emulsions in contributing to the development of reduced-fat products and as vehicles for the delivery of nutrients, the potential of double emulsions in the food technology has yet to be fully elucidated (Dickinson, 2011).

The amount of fat in functional foods is a relevant factor for nutritional purposes and also its fatty acid profile. The lack of differences in the fatty acid profile of all emulsions during the treatment (0-48 h, 65°C) pointed out that the nutritional value of the oil was not affected by the heat treatment, regardless of the type of emulsion.

Furthermore, both antioxidants were effective in keeping the supply of the main fatty acids in both types of emulsions, α-linolenic and oleic acid in linseed oil and olive oil emulsions, respectively. Consequently, irrespective of the type of emulsion and antioxidant used, both oils provided an interesting fatty acid profile, leading to excellent ratios from the nutritional standpoint.

García Iñiguez de Ciriano et al. (2010) in a previous work concluded about the need of the use of antioxidants in highly unsaturated O/W emulsions in order to control lipid oxidation. Regarding the oxidation status, a distinct behaviour through the accelerated oxidation study was noticed between the two oils, as it was expected, due to their different profile. Thus, their response to the two antioxidants and the type of emulsion applied was studied independently. Whereas olive oil emulsions tend to remain unaltered during heating irrespective of the antioxidant and type of emulsion applied, linseed oil emulsions were more sensitive to these two variables under study. Discussion of data will take into account these relevant aspects.

4.1. Olive oil emulsions

In olive oil emulsions, the analysis of primary oxidation products pointed out that PV remained, in every condition, below the maximum acceptable level by the Commission Regulation (EC) No 1989/2003 set at <20 meq O_2/kg oil. Dienes increased linearly during storage in all cases, without differences between antioxidants during the heating treatment. Trienes and TBARS, however, showed higher values for W/O/W emulsions compared to O/W ones. This finding can indeed be attributed to the different procedures applied to obtain the emulsions. The preparation of the W/O/W emulsions includes a 70°C heating treatment of the oil, which could have contributed to promote a slight degree of lipid oxidation.

It is worthy to mention that in general CD, CT and TBARS are inherently insensitive to MUFA, as oleic acid hydroperoxides contain less than two double bonds. In general, significant amounts of TBARS are only formed when fatty acids with 3 or more double bonds are involved (Fennema, 1996). This fact could lead to underestimation of oxidation in highly monounsaturated lipids (Waraho, Cardenia, Decker & McClements, 2010). In our study, these three parameters were able to reveal differences among emulsions.

Unexpectedly, the α-TOH content did not decrease during the accelerated oxidation study of olive oil emulsions, moreover a slightly increase of the content was noticed in the case of O/W emulsions. It could be related to a synergism process among different antioxidants, between α-TOH and polyphenols in which olive oil is rich. A number of studies describe a synergic effect between α-TOH and polyphenols (Zhu, Huang, Tsang & Chen, 1999; Pedrielli & Skibsted, 2002), whereby the regeneration of α-TOH from its one-electron-oxidized form by some flavonoids was suggested, in analogy to the well-known synergism between α-TOH and ascorbate (Niki, 1987; Bisby & Parker, 1995). This process could have also occurred in O:W/O/W emulsions, but it would have been masked by the fact that these emulsions were subjected to higher oxidative processes, which are more antioxidant demanding.

However, total antioxidant capacity measured by L-ORAC, showed a slight decrease in both types of emulsions (O/W and W/O/W), probably in response to the increased oxidation trend

that was observed during heating by dienes and trienes analysis. It points to the fact that the total antioxidant capacity of olive oil is dependent on other types of compounds, different from α-TOH, which was hardly modified during heating. In fact, phenolic compounds greatly contribute to olive oil stability, even more than other compounds (Pellegrini, Visioli, Buratti & Brighenti, 2001; Farhoosh, Sharif & Rafire, 2011). The presence of tyrosol, hydroxytyrosol and catechol has been well described in olive oil (Choe & Min, 2009), and a high polyphenol concentration was detected in the olive oil used in this work (253.7 mg gallic acid/kg oil).

4.2. Linseed oil emulsions

As previously stated, linseed oil containing emulsions were far more sensitive than olive oil emulsions with respect to the use of different antioxidants and emulsion system during the accelerated oxidation study.

Although the PV and TBARS pointed out a favorable initial oxidation state compared to olive oil, a rapid increase in these parameters was observed during the storage due to the high content in polyunsaturated fatty acids, which are more likely to form hydroperoxides. It has been reported that the rate of oxidation of α-linolenic and linoleic acid is 20 and 10-fold the value exhibited by oleic acid, respectively (Fennema, 1996). Furthermore, a positive correlation was found between PV and CD (R^2=0.915), in agreement to the results found by Marmesat, Morales, Velasco, Ruiz-Mendez and Dobarganes (2009). The water *Melissa* extract was far more effective in lagging the increase in PV in O/W, whereas BHA was more efficient in W/O/W emulsions. *Melissa* extract is rich in rosmarinic acid (García-Iñiguez de Ciriano et al., 2010), a polar molecule known by its antioxidant activity, which effectively delays the formation of oxidation products in O/W emulsions. As a chain-breaking antioxidant, it maintained a lag-phase during which the substrate was not substantially oxidized, that continued until the antioxidant is completely consumed. However BHA since it is placed in the oily phase, due to its lipophilic character, was not able to do so on the O/W emulsions.

In the case of W/O/W emulsions it was only after 48h heating when there was a different behaviour among both antioxidants. BHA was dispersed in the water internal phase and it is hypothesised that is slowly released into the oily phase. As the oil phase of W/O/W emulsion is commonly regarded as a liquid membrane separating the internal and external aqueous phases (Dickinson, 2011), BHA could efficiently exert its antioxidant activity in the oily phase along its slow release. In this case, *Melissa* extract rich in rosmarinic acid, exerts directly its antioxidant activity in the oily/aqueous interface. A linear increment of TBARS and PV was observed from the first day of heating until the end. This finding confirmed the effectiveness of *Melissa* extract protecting the oil droplets when the antioxidant is located in the aqueous environment.

α-TOH and L-ORAC data at initial time were significantly higher in linseed oil compare to olive oil containing emulsions (2 fold and 4 fold, respectively). The increasing oxidation status shown by primary and secondary oxidation products during heating was reflected in a decrease of L-ORAC, particularly evident in *Melissa* containing emulsions. However, the tocopherol loss during the treatment was hardly appreciated. Thus, it seemed that the compounds that accounted for the main L-ORAC activity were different from tocopherol, which should be confirmed in further experiments.

5. CONCLUSIONS

Olive oil emulsions remained unaltered during a 48h accelerated oxidation study and a low influence of the type of antioxidant and location was detected. Linseed oil emulsions showed a high initial antioxidant status, although they were significantly affected by the accelerated oxidation study. In that case, differences were found in the effectiveness of the used antioxidants depending on the emulsions: the hydrophilic antioxidant (*Melissa*) was more efficient in O/W emulsions, whereas the lipophilic antioxidant (BHA) was more effective in W/O/W ones. No clear distinction was observed for the stability of W/O/W emulsions compared to O/W ones for each antioxidant used.

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