Effects of caffeic acid and bovine serum albumin in reducing the rate of development of rancidity in oil-in-water and water-in-oil emulsions

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Effects of Caffeic Acid and Bovine Serum Albumin in Reducing the Rate of Development of Rancidity in Oil-in-water and Water-in-oil Emulsions

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ABSTRACT

The antioxidant properties of caffeic acid and bovine serum albumin in oil-in-water and water-in-oil emulsions were studied. Caffeic acid (5 mmol/kg emulsion) showed good antioxidant properties in both 30% sunflower oil-in-water (OW) and 20% water-in-sunflower oil emulsions (WO), pH 5.4, during storage at 50 ºC. Although bovine serum albumin (BSA) (0.2%) had a slight antioxidant effect, the combination of caffeic acid and BSA showed a synergistic reduction in the rate of development of rancidity, with significant reductions in concentration of total volatiles, peroxide value (PV) and p-anisidine value (PA) for both emulsion types. The synergistic increase in stability of the OW and WO emulsions containing BSA and caffeic acid was 102.9 and 50.4 % respectively based on TOTOX values, which are calculated as 2PV + PA, with greater synergy calculated if based on formation of headspace volatiles, The OW emulsion was more susceptible to the development of headspace volatiles by oxidation than the WO emulsion, even though the degree of oxidation assessed by the TOTOX value was similar.

Keywords: Antioxidant activity; Bovine serum albumin; Caffeic acid; Emulsions; Oxidative stability

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48

49 **1. INTRODUCTION**

50 Lipid oxidation is of great concern to the consumer because it causes not only changes in the quality
51 attributes of foods, including taste, texture, shelf life, appearance and flavor, but also due to the strong
52 decrease in the nutritional value and safety caused by the loss of antioxidants and the formation of
53 harmful components including free radicals and reactive aldehydes (Halliwell, Murcia, Chirico &
54 Aruoma, 1995).

55 Oxidation in food emulsions is of particular interest since oil is widely consumed in foods which are
56 either water-in-oil, for example, butter and margarine, or oil-in-water emulsions, for example,
57 mayonnaise, milk and cream. Lipid oxidation in emulsions is generally recognized as being more
58 complex than lipid oxidation in bulk oil systems, as the emulsification process will lead to the
59 formation of a large interfacial area, and lipid oxidation is initiated at the interface between oil and
60 water, where different non-polar and polar compounds in the system can interact (Frankel, 1998;
61 McClements & Decker, 2000).

62 One of the most effective methods of retarding lipid oxidation in fatty foods is to incorporate
63 antioxidants. The behavior of antioxidants is also more complex in emulsions than in bulk oil since
64 more variables are involved, including the emulsifier and pH (Mancuso, McClements & Decker, 1999;
65 SØrensen et al., 2008). Antioxidant efficacy in food systems depends on various factors including the
66 structure (i.e., number and position of hydroxyl groups bound to the aromatic ring and presence of
67 other substituents), chemical reactivity of the phenolics, interactions with food components, and
68 environmental conditions. The partitioning of the antioxidant into the different phases is an important
69 factor for emulsions (Decker, Warner, Richards & Shahidi, 2005). The efficacy of antioxidants in bulk
70 oil and in dispersed systems is affected by their polarity as described by the “polar paradox” (Frankel,
71 Huang, Kanner & German, 1994). According to the polar paradox, polar antioxidants like ascorbic

acid and Trolox are more active in non-polar media like bulk oils than less polar components including ascorbyl palmitate and tocopherol. These apparent paradoxical effects of the antioxidants have been reported as being due to the polar antioxidants being located at the air-oil interface in bulk oils, i.e. situated where oxidation is suggested to take place or at least to be initiated. In addition, they may act in non-polar media by chelation of metal ions. On the other hand, lipophilic antioxidants are more active in polar systems, because they are located at the oil-water interface where oxidation is propagated (Frankel et al, 1994; Huang, Hopia, Schwarz, Frankel & German, 1996; Porter, 1993).

Phenolic compounds, such as caffeic acid, have received increasing interest due to their antioxidant activity, which improves the stability of lipid-containing foods and their possible beneficial effects on human health. Caffeic acid may exert its antioxidant effect by donating a hydrogen atom to free radicals, thereby acting as chain-breaking antioxidants, or may act as a metal chelator, which reduces the activity of prooxidants (Gülçin, 2006; Leonardis & Macciola, 2003).

Although antioxidants have been frequently studied in oils, emulsions, and other foods, there have been few reports of how proteins, which are commonly present in foods, may affect the activity of antioxidants. Most antioxidants of interest for foods have one or more phenolic hydroxyl groups, and several studies have demonstrated that molecules with this structure may bind to proteins. Polyphenols may associate with proteins through hydrophobic interactions and hydrogen bonding (Oda, Kinoshita, Nakayama & Kakehi, 1998), and a range of phenolic antioxidants has also been shown to bind to bovine skin proteins (Wang & Goodman, 1999).

Proteins have been shown to have weak antioxidant activity including both metal chelating and radical scavenging activity (Arcan & Yemenicioğlu. 2007). Bovine serum albumin **(**BSA), a minor whey protein with M.W. 66 kDa has surface-active properties and has been used to stabilize model food emulsions (Rampon, Lethuaut, Mouhous-Riou & Genot, 2001).

The aim of this study was to determine the influence of BSA on the total antioxidant activity of caffeic acid in model food emulsions. The pH of the emulsions was 5.4, which is a typical pH for margarine samples.

2. MATERIALS AND METHODS

2.1. Chemicals

All solvents used were analytical or HPLC grade (Merck & Co. Inc., Darmstadt, Germany). Isooctane, glacial acetic acid, ferrous sulfate, barium chloride, ammonium thiocyanate, polyoxyethylene sorbitan monolaurate (Tween-20), p-anisidine, cumene hydroperoxide, bovine serum albumin (BSA), hexanal, and bromobenzene were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). Refined sunflower oil was purchased from a local retail outlet. Fatty acids were purchased from Sigma Chemical Co. (Saint Louis, MO, USA).

The fatty acid composition of the sunflower oil is shown in **Table 1**.

2.2. Removal of tocopherols from sunflower oil

Tocopherols were removed from sunflower oil by column chromatography using activated alumina as described by Yoshida, 1993.

2.3. Emulsion preparation

Oil-in-water emulsions (30% oil) were prepared by dissolving Tween-20 (1%) in acetate buffer (0.1 M, pH 5.4), either with or without BSA (0.2%) and caffeic acid (5 mmol/kg emulsion). Water-in-oil emulsions (20% water) were prepared by dissolving Dimodan® (1%) in acetate buffer (0.1 M, pH 5.4), either with or without BSA (0.2%) and caffeic acid (5 mmol/kg emulsion). Emulsions were prepared by the dropwise addition of oil to the water phase, with sonication by a Vibracell sonicator (Sonic & Materials, Newton, CT, USA) whilst cooling in an ice bath for 5 min.

2.4. Emulsion oxidation

All emulsions were stored in triplicate in 25 mL glass bottles in the dark (inside an oven) at 50 ºC, and each replicate was analysed once.

Aliquots of each emulsion were removed periodically for peroxide value (PV), *p*-anisidine value (PA) determinations and for analysis of volatile oxidation products by gas chromatography (GC).

2.5. Determination of fatty acid composition

The fatty acid composition of sunflower oil was determined by GC on an HP Agilent 6890N chromatograph equipped with a HP 7683 injector and a flame ionization detector (FID). Fatty acid methyl esters (FAMEs) were prepared by transesterification according to Shehata, de Man & Alexander, 1970. Chromatographic separation was carried out using a SP-2560 column (100 m length, 0.25 mm ID and 0.20 µm film thickness; Supelco). The oven temperature was 120 ºC for 5 min, followed by temperature programming to 170 ºC at 2 ºC min^{-1}, 200 ºC at 5 ºC min^{-1}, and then increased to 235 ºC at 2 ºC min^{-1}. Helium was used as carrier gas, the FID temperature was 280 ºC and the injection port was held at 260 ºC.

2.6. Spectrophotometric determination of peroxide value (PV)

Emulsion (0.3 mL) was added to isooctane/2-propanol (3:2 v/v, 1.5 mL) and the mixture was mixed on a vortex mixer three times for 10 s each time. After centrifugation for 2 min at 1000 x g, the clear upper layer (0.2 mL) was collected and peroxides were quantified using a method based on that of Díaz, Dunn, McClements & Decker, 2003. Lipid peroxide concentration was determined using a cumene hydroperoxide standard curve (r^2=0.9977).

2.7. Spectrophotometric determination of p-anisidine Value (PA)

The PA value was determined by AOCS Official Method no. cd 18-90, 1989.

2.8. Solid phase microextraction (SPME) sampling conditions

An aliquot of emulsion (0.2 g) was weighed in a 2mL vial. A magnetic follower was added and the vial was capped with a Teflon-faced rubber septum and plastic cap. The vial was placed in a water bath on a magnetic stirrer and the sample was equilibrated for 2 min at 60ºC. The septum was

144 manually pierced with the SPME needle and the fiber was exposed to the emulsion headspace for 60
145 min and transferred to the gas chromatograph where the volatiles were desorbed in the injection port.
146 The desorption time in the injection port was 15 min.

147 ***2.9. SPME/GC analysis***

148 Volatile oxidation compounds were monitored by headspace analysis with solid phase microextraction
149 (HS-SPME). A manual SPME fiber holder unit and 30 µm DVB-CAR-PDMS fiber (Sigma-Aldrich
150 Company Ltd., Dorset, UK) were used to adsorb volatiles from the emulsion in a closed vial at 60ºC
151 with a sampling time of 60 min. GC analyses were performed with a HP 5890 series II gas
152 chromatograph (Agilent UK, South Queensferry, UK) equipped with FID detector and split/splitless
153 injector. Chromatographic separation was carried out using a HP-5-column (15 m length, 0.25 mm ID
154 and 0.25 µm film thickness; Agilent UK). The oven temperature was 40 ºC for 10 min, followed by
155 temperature programming to 140 ºC at 2.5 ºC min^{-1}, and then increased to 300 ºC at 20 ºC min^{-1}.
156 Helium was used as carrier gas in the splitless mode. The FID temperature was 280 ºC and the
157 injection port was held at 260 ºC.

158 The identification of all compounds was based on the mass spectra determined by GC-MS using a HP
159 5890 series II gas chromatograph with MS detector and by comparison of their retention time with
160 those of authentic standards. Retention times for a series of n-alkanes (C_5-C_{25}) were determined and
161 used to calculate the Linear Retention Indices (LRI values) of detected compounds.

162 Bromobenzene (1µL/g emulsion) was added as an internal standard prior to analysis. The relative
163 concentrations of the investigated compounds were calculated by relating the area of the internal
164 standard to the area of the compound of interest, defined as:

165 $$\text{Relative conc.} = \frac{\text{Peak area of particular compound}}{\text{Peak area of IS}} \text{x IS conc.}$$

166 ***2.10. Confocal microscopy***

Confocal microscopy was performed with a Leica TCS SP2 AOBS confocal laser scanning microscope mounted on a Leica inverted DM IRE2 microscope for the oil-in-water emulsions and with a Leica TCS SP5 confocal laser microscope for the water-in-oil emulsions.. A Nile Red/Nile Blue mixture (0.01%) was added to an emulsion sample and examined using x10, x20 and x 40 objectives. The image was obtained by exciting the sample with a 633nm Helium-Neon laser with an emission band of 630-750nm.

2.11. Calculation of synergy

Synergy was calculated by comparing the times to a given level of deterioration, defined as induction period for the calculation, as described in Almajano & Gordon, 2004.

$$\%\text{synergism} = 100\,\frac{\left[\text{IP}(a+p) - \text{IP}(c)\right] + \left[(\text{IP}a - \text{IP}c) + (\text{IP}p - \text{IP}c)\right]}{\left[(\text{IP}a - \text{IP}c) + (\text{IP}p - \text{IP}c)\right]}$$

where IP= induction period, *a*= antioxidant, *p* = protein and *c* = control.

2.12. Statistical analysis

The triplicate determinations were used to calculate the mean (m), standard deviation (sd), and standard error of the mean (SEM).

Times to selected levels of deterioration were compared, and significant differences at the 5% level were assessed by one-way ANOVA using commercial software (Statistica 6.0). To verify the association among experimental data, Pearson correlation analysis was performed using the same statistical package; p-values < 0.05 were considered significant.

3. RESULTS AND DISCUSSION

189 The sunflower oil used contained linoleic acid (64.17%), and oleic acid (25.01%) as the main
190 unsaturated fatty acids, with linolenic acid being present at 0.16% (**Table 1**).
191 The emulsions were stored at 50 ºC to accelerate oxidative changes that would occur more slowly, at
192 lower temperatures. Reproducibility of the data was good with the SEM being <25% for 80% of the
193 samples analysed by PV, and the SEM being <25% for 90% of the samples analysed by PA.

194 The initial PV of the emulsion samples was similar with values of 0.05 mM cumene hydroperoxide for
195 OW emulsions and 0.07-0.17 mM for WO emulsions. After 14 days of oxidation the OW emulsions
196 reached significantly different PV values in the order: OW > OW-BSA > OW-CAF > OW-CAF-BSA.
197 The PV values reached in WO emulsions were in the same order up to 35 days but the PV of WO-
198 CAF was higher than WO-BSA at 42 days, since the PV of the control and BSA samples declined
199 sharply after 35 days with corresponding sharp increases in PA values indicating degradation of the
200 hydroperoxides.

201 As shown in **Figure 1**, the maximum PV reached was 10.43 mM hydroperoxide at 42 days for the OW
202 sample, followed by 7.72 mM hydroperoxide for OW-BSA. In WO emulsions the maximum value
203 was reached at 35 days for the WO sample followed by the WO-BSA sample (6.28 and 5.44 mM
204 hydroperoxide, respectively); then there was a decrease in PV for all WO emulsions as the rate of
205 hydroperoxide formation became less than hydroperoxide decomposition.

206 At 35 days of storage, the extent of formation of hydroperoxides in the OW-CAF and WO-CAF
207 samples was lower than in the appropriate control sample with inhibition of 66.7 and 34.7 %,
208 respectively, indicating the antioxidant effect of caffeic acid. The greater effect in the OW emulsion
209 may be due to type of emulsion, the higher caffeic acid:oil ratio (16.7 mmol/kg oil) in the OW
210 emulsion, which was 2.7 times higher than in the WO emulsion (6.25 mmol/kg oil) and the difference
211 between oil concentration in the emulsions. BSA increased the activity of caffeic acid in both
212 emulsions.

213

The PA value determination, a measure of secondary oxidation products (List, Evans, Kwolek, Warner, Boundy & Cowan, 1974), confirmed the findings of the PV analysis. The PA test was used to determine the level of aldehydes, principally 2-alkenals and 2,4-alkadienals, present in the emulsions. The PA values were relatively stable for the samples with caffeic acid, and with both caffeic acid and BSA throughout the study.

As shown in **Table 2**, in the presence of BSA, the stability of the OW emulsions containing caffeic acid increased by more than 200%, when assessed by the time to PA = 0.15, but BSA did not contribute any significant increase in stability to WO emulsions containing caffeic acid when assessed by the PA values. The TOTOX value is a measure of both primary and secondary oxidation products, where TOTOX = 2PV + PA. Synergy between the caffeic acid and bovine serum albumin, in retarding oxidation of emulsions, was investigated by consideration of the TOTOX values. As shown in **Table 2**, the OW sample took 8.19 days to reach a TOTOX value of 3.7, followed by OW-BSA with 10.72 days, then OW-CAF with 23.7 days and finally OW-CAF-BSA, the most stable, with 42 days. The WO emulsions showed similar times to reach a TOTOX value of 8.9.

Headspace solid phase microextraction (HS-SPME) was used to isolate the headspace volatile products formed during secondary oxidation of OW and WO emulsions. Volatile aldehydes have great importance as oxidation products because of their contribution to the aroma of oxidised oils and emulsions. Hydrophobic flavor components can be perceived at lower concentrations in water than in oil, since many of the lipid oxidation products have higher solubility in the oil phase. This means that, for a fixed concentration of volatile components, their concentration in the headspace of an emulsion decreases as the oil concentration increases. As a consequence, a low fat emulsion may be perceived as more oxidized than a high fat emulsion, even though both emulsions have the same concentration of volatile components.

Figure 2 shows the change in concentration of hexanal and total volatile products with time expressed as relative peak area. Hexanal has been widely used as a marker of volatile oxidation products of n-6 fatty acids in previous studies (Rouseff & Cadwallader, 2001). In both systems, hexanal was the main volatile formed during storage. The OW emulsion was more susceptible to headspace volatile formation than the WO emulsion, as shown by the considerably higher concentrations of total volatiles that were formed, even though the degree of oxidation assessed by the TOTOX value was not significantly different in the two emulsion types (**Table 3**). At day 42, the TOTOX value for the OW emulsion containing caffeic acid and BSA was even lower than that of the analogous WO emulsion. The difference in headspace volatile concentration is partly due to the reduction of vapour pressure of each volatile component in the greater mass of oil present in the WO emulsions, since, according to Raoult's law, the vapour pressure of a solute is proportional to its molar concentration. However, differences in the relative importance of hydroperoxide decomposition pathways into volatile and non-volatile products may also occur in the different emulsion types, since the headspace volatile: PA ratio after 42 days storage was 6.7-7.9 times higher in the OW emulsion than in the corresponding WO emulsion, for samples containing no additive, BSA or caffeic acid and 49.3 times higher for the samples containing caffeic acid and BSA (Table 3). The higher oxidative susceptibility of OW emulsions has been reported extensively, and it is attributed to the greater extent of interfacial interactions that are possible between the lipid substrate and prooxidants including metal ions in the aqueous phase as a consequence of the greater surface area presented by the emulsion droplets (McClements & Decker, 2000; Jacobsen, Let, Nielsen & Meyer, 2008). It has been suggested that lipid oxidation in WO emulsions will occur at a rate similar to that in bulk oils because the surface of the lipid phase is exposed directly to air (McClements & Decker, 2000), but effects of antioxidants, metal ions and chelating agents will differ in these media. In WO emulsions, caffeic acid inhibited the formation of hexanal and total volatile compounds at the end of the storage period by 75.48 and 53.51 %, respectively. Caffeic acid showed good antioxidant properties in OW emulsions inhibiting the formation of hexanal and total volatile compounds by 95.08 and 87.27 %, respectively. BSA increased

the antioxidant activity of caffeic acid in inhibiting the formation of hexanal and volatile compounds in the later stages of storage in WO emulsions (by 18.49 and 26.49 %, respectively), but was less effective in OW emulsions (2.51 and 3.32 %, respectively).

The synergistic increase in stability of the OW and WO emulsions prepared with BSA containing caffeic acid was 277.34 and 468.53 %, respectively, based on the time for the total volatile concentration to reach 585.37 and 364.56 μg bromobenzene/ g oil, respectively (**Table 2**).

Positive correlations between PV and total volatiles (r=0.892) and also between PA and both hexanal and total volatiles (r=0.992, r=0.981, respectively) in OW emulsions were found. Weaker correlations among parameters analyzed were found in WO emulsions (**Table 4**). Faster hydoperoxide decomposition compared to hydroperoxide formation in the WO emulsions, which leads to a reduction in PV but an increase in total volatiles, contributes to the weaker correlation between PV and total volatiles in this emulsion system.

In the secondary oxidation stage, volatile compounds (e.g. alcohols and aldehydes) are formed by the decomposition of lipid hydroperoxides. In particular, volatile aldehydes have a great importance as an indicator of oxidation due to their considerable contribution to the aroma and flavor deterioration of the final products. The concentration of volatile compounds identified in OW and WO emulsions at 21 and 42 days of oxidation is summarized in **Table 5.** The profile of volatiles was dominated by oxidation products derived from linoleic acid. As confirmed by GC-MS analysis, hexanal, *(E)*-2-heptenal, *(E)*-2-octenal and *(E,E)*-2,4-decadienal were the major volatile products in the autoxidation, while pentanal, 1-octen-3-ol, nonanal and *(E)*-dec-2-enal were also present in significant amounts. Hexanal, *(E)*-2-octenal, 2-heptenal, pentanal,and *(E,E)*-2,4-decadienal are major oxidation products of linoleic acid (Selke, Rohwedder & Dutton, 1980; Frankel, 1998; Aidos, Jacobsen, Jensen, Luten, Van der Padt & Boom, 2002; Jiménez-Álvarez, Giuffrida, Golay, Cotting, Destaillats, Dionisi et al, 2008), whereas nonanal, octanal, 1-heptanol, 1-octanol, and *(E)*-dec-2-enal are formed from triacylglycerols containing oleic acid (Selke et al, 1980).

289 The combination of caffeic acid and BSA was most effective at reducing the formation of both
290 hexanal and total volatiles in both emulsion systems. Caffeic acid was very effective at reducing
291 hexanal and total volatile formation in the OW emulsion, but its effect was weaker in the WO
292 emulsion where it was no better than BSA in reducing total volatile formation up to 35 days. This is
293 consistent with the increased hydroperoxide decomposition observed in the WO emulsion containing
294 caffeic acid (Figure 1b). The relative concentrations of the volatiles changed to some extent during the
295 oxidation with E-2-octenal becoming a major volatile product at later stages of oxidation in the OW
296 emulsion.

297 The antioxidant power and other important biological properties of caffeic acid are well substantiated
298 (Gülçin, 2006; Yanishlieva & Marinova, 1995; Chen & Ho, 1997). Caffeic acid retarded the formation
299 of hydroperoxides in menhaden oil-in-water emulsions (Maqsood & Benjakul, 2010), and De
300 Leonardis & Macciola (De Leonardis & Macciola, 2003) reported that the antioxidant effectiveness of
301 caffeic acid was better than that of BHA in hydrophobic phases such as cod liver oil.

302 The interactions between polyphenols and albumin have attracted much attention due to their
303 importance in food (He, Liang, Wang & Luo, , 2010; Prigent, Voragen, Visser, Van Koningsveld &
304 Gruppen, 2007). Caffeic acid is known to bind to BSA during storage in air with antioxidant activity
305 being incorporated in the protein fraction (Bartolomé, Estrella & Hernández, 2000; Almajano &
306 Gordon, 2004; Almajano, Carbo, Delgado & Gordon, 2007) but this does not occur in samples stored
307 under nitrogen (unpublished data). Confocal microscopy showed that BSA was present at the interface
308 between the oil and water phases in both an oil-in-water emulsion (Figure 3a, b), and a water-in-oil
309 emulsion (c, d). BSA can be seen clearly since it enhances the nile blue fluorescence at 670 nm (Lee,
310 Suh & Li, 2003). Hence, it can be deduced that caffeic acid is oxidized to a quinone, and binds to BSA
311 by reaction with amine groups in the side chains of amino acids such as lysine, as described by Rawel,
312 Rohn, Kruse & Kroll (2002). This transfers the caffeic acid from homogeneous solution in the aqueous
313 phase to the interface where it is more effective as an antioxidant. The caffeic acid retains a free
314 phenolic group, which is activated by a positively charged nitrogen atom as shown in scheme 1.

CONCLUSIONS

Development of headspace volatiles by lipid oxidation was significantly affected by emulsion type. The oil-in-water emulsions had a higher headspace volatile concentration, despite the degree of total oxidation assessed by the TOTOX value being similar to that of the water-in-oil emulsions. Although BSA had a slight antioxidant effect, it was found that BSA exerted a synergistic effect with caffeic acid in both emulsion types, and this is consistent with previous reports of synergy accompanied by formation of a protein-antioxidant adduct during storage (Almajano & Gordon, 2004). BSA caused a greater synergistic increase in the antioxidant activity of caffeic acid in a water-in-oil emulsion than in an oil-in-water emulsion.

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Table 1. Fatty acid composition of sunflower oil

Fatty acid name		**Amount (%)**
Saturated (S)		*10.42*
Myristic acid	C14:0	0.11 ± 0.02
Palmitic acid	C16:0	5.72 ± 0.04
Margaric acid	C17:0	0.05 ± 0.00
Stearic acid	C18:0	3.49 ± 0.05
Arachidic acid	C20:0	0.24 ± 0.00
Behenic acid	C22:0	0.57 ± 0.01
Tricosanoic acid	C23:0	0.05 ± 0.00
Lignoceric acid	C24:0	0.19 ± 0.01
Unsaturated (U)		*89.58*
Monounsaturated		*25.25*
Palmitoleic acid	C16:1	0.10 ± 0.00
Oleic acid	C18:1(n-9)	25.01 ± 0.63
Eicosenoic acid	C20:1(n-9)	0.14 ± 0.00
Polyunsaturated		*64.33*
Linoleic acid	C18:2(n-6)	64.17 ± 0.73
Linolenic acid	C18:3(n-3)	0.16 ± 0.00
S : U		*0.12*
Omega 6		*64.17*
Omega 3		*0.16*
Omega 6 : Omega 3		*401.06*

Table 2. Times in days for oil-in-water and water-in-oil emulsions to reach indicated values at 50 ºC

Oil-in-water emulsion (value at assessment time)	PV (1.79 mM hydroperoxide)	PA (0.15)	TOTOX (3.7)	Hexanal (14.63 μg bromobenzene / g oil)	Total volatiles (585.37 μg bromobenzene / g oil)
OW	9.95	2.65	8.19	2.36	7.54
OW-BSA	11.22	8.84	10.72	6.12	10.99
OW-CAF	24.91	9.41	23.7	22.49	13.22
OW-CAF-BSA	42.00	42.00	42.00	42.00	42.00
% Synergy	97.47	202.60	102.9	65.95	277.34
Water-in-oil emulsion (value at assessment time)	PV (2.15 mM hydroperoxide)	PA (4.59)	TOTOX (9.5)	Hexanal (18.71 μg bromobenzene / g oil)	Total volatiles (364.56 μg bromobenzene / g oil)
WO	12.87	17.34	16.2	3.95	13.42
WO-BSA	15.82	17.26	17.7	13.77	15.84
WO-CAF	24.47	39.47	27.2	17.70	16.02
WO-CAF-BSA	42.00	42.0	35	42.00	42.00
% Synergy	100.20	12.10	50.4	61.49	468.53

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441 **Table 3.** PV, PA, TOTOX and relative concentration of total volatiles for emulsions at 42 days

	PV (mM hydroperoxide)	PA	TOTOX	Total volatiles (μg bromobenzene / g oil)	Total volatiles: PA ratio
OW	10.43±1.97	17.29±1.68	38.15	6219.58±999.53	359.72
OW-BSA	7.72±1.76	11.77±1.94	27.21	5477.40±675.16	465.37
OW-CAF	4.82±0.03	0.71±0.12	10.35	791.53±48.42	1114.83
OW-CAF-BSA	1.79±0.32	0.15±0.04	3.73	585.40±48.15	3902.67
WO	3.97±0.33	34.03±0.65	41.97	1822.68±279.83	53.56
WO-BSA	2.87±0.60	23.49±2.11	29.23	1379.19±8.32	58.71
WO-CAF	3.42±0.34	5.11±0.08	11.95	847.29±95.04	165.81
WO-CAF-BSA	2.15±0.24	4.59±0.91	8.89	364.56±14.21	79.42

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Table 4. Significant Pearson´s correlations among parameters analyzed (PV, PA, hexanal and total volatiles)

	Oil-in-water emulsion				*Water-in-oil emulsion*			
Pearson´s correlation:	PV	PA	Hexanal	Total volatiles	PV	PA	Hexanal	Total volatiles
PV	-	0.856	0.860	0.892	-	0.504	0.712	0.796
PA	0.856	-	0.992	0.981	0.504	-	0.904	0.885
Hexanal	0.860	0.992	-	0.987	0.712	0.904	-	0.922
Total volatiles	0.892	0.981	0.987	-	0.796	0.885	0.922	-

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476 **Table 5.** Concentration (expressed as equivalent to bromobenzene) of volatile compounds identified in

477 oil-in-water and water-in-oil emulsions during storage at 50 ºC

Compound	Concentration (µg bromobenzene / g oil)			
	OW (21 days)	OW (42 days)	*WO (21 days)*	*WO (42 days)*
Butanal	20.97	46.74	6.36	24.05
Pentanal	15.19	71.97	14.69	45.71
(E)-2-Pentenal	18.39	141.37	nd	nd
1-Pentanol	5.91	88.85	3.37	28.61
Hexanal	70.66	606.9	61.7	310.26
2-Hexenal	nd	20.44	0.83	5.43
2-Heptanone	nd	10.95	1.25	7.38
Heptanal	nd	60.42	1.75	14.36
(E)-2-Heptenal	17.54	259.35	12.96	53.07
1-Heptanol	2.12	11.12	1.72	3.61
1-Octen-3-ol	5.93	117.9	3.64	15.92
2-Pentyl-furan	nd	nd	9.13	34.28
Octanal	8.36	65.37	2.18	16.81
3-Octen-2-one	2.35	28.01	1.53	14.08
5-Ethyldihydro-2(3H)-furanone	nd	nd	13.39	116.33
(E)-2-Octenal	15.08	550.12	2.89	28.28
1-Octanol	1.54	14.92	1.37	4.04
Heptanoic acid	2.99	10.37	0.87	7.23
2-Nonanone	5.18	43.57	4.07	20.49
Nonanal	10.84	26.5	3.93	13.61
(E,E)-2,4-Octadienal	0.07	5.81	nd	nd
3-Nonen-2-one	0.55	15.27	0.47	2.27
(E)-2-Nonenal	1.69	18.45	2.48	13.36
2-Decanone	0.91	13.77	1.07	2.67
(E,E)-2,4-Nonadienal	3.02	67.91	3.1	15.89
3-Methylbut-2-enal	11.63	85.71	3.02	9.98
Methyl-cyclodecane	1.28	7.55	0.55	2.07
(E)-2- Decenal	7.11	70.53	3.71	14.04
Nonanoic acid	1.35	32.75	1.11	7.45
(E,E)-2,4-Decadienal	70.46	443.56	19.32	60.09
5-pentyl-5(H)-furan-2-one	6.25	229.94	3.79	40.06
(E)-2-Tridecenal	7.18	75.28	3.48	11.95
6-Dodecanone	1.16	34.52	1.06	3.1
2-Butyloct-2-enal	8.14	100.88	2.51	7.71
Total volatiles	323.85	3376.8	193.3	954.19

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FIGURE LEGENDS

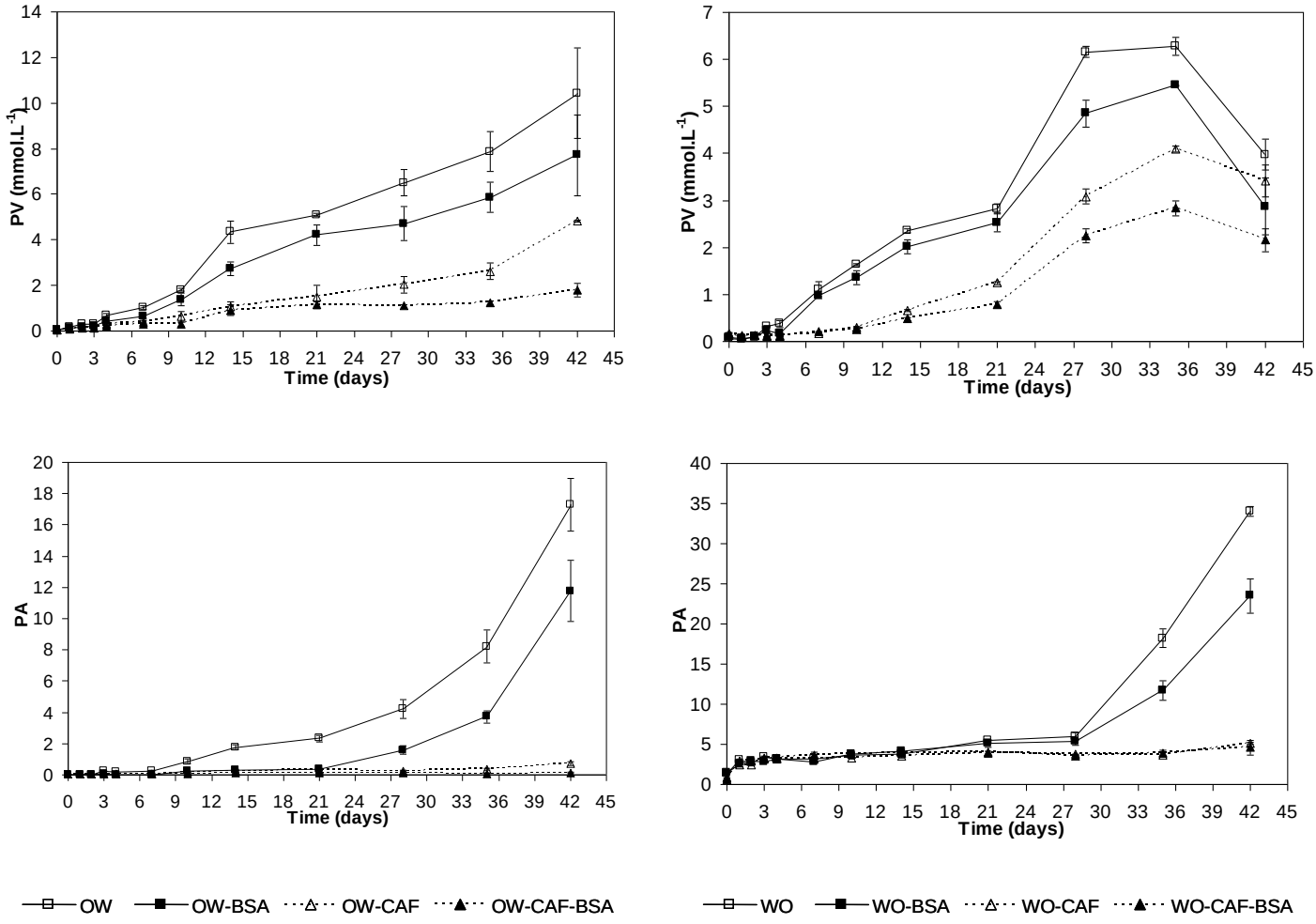
Figure 1. Changes in peroxide value and *p*-anisidine value of a) OW emulsions and b) WO emulsions containing antioxidants during storage at 50 ºC.

Figure 2. Changes in hexanal and total volatiles concentration of a) OW emulsions and b) WO emulsions containing antioxidants during storage at 50 ºC, with concentration expressed as area equivalent to bromobenzene.

Figure 3. Oil-in-water emulsions stained with oil red- nile blue mixture (a) with BSA; (b) no protein

A) Oil-in-water emulsion

B) Water-in-oil emulsion



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508 **Figure 1.** Changes in peroxide value and *p*-anisidine value of a) OW emulsions and b) WO emulsions

509 containing antioxidants during storage at 50 ºC

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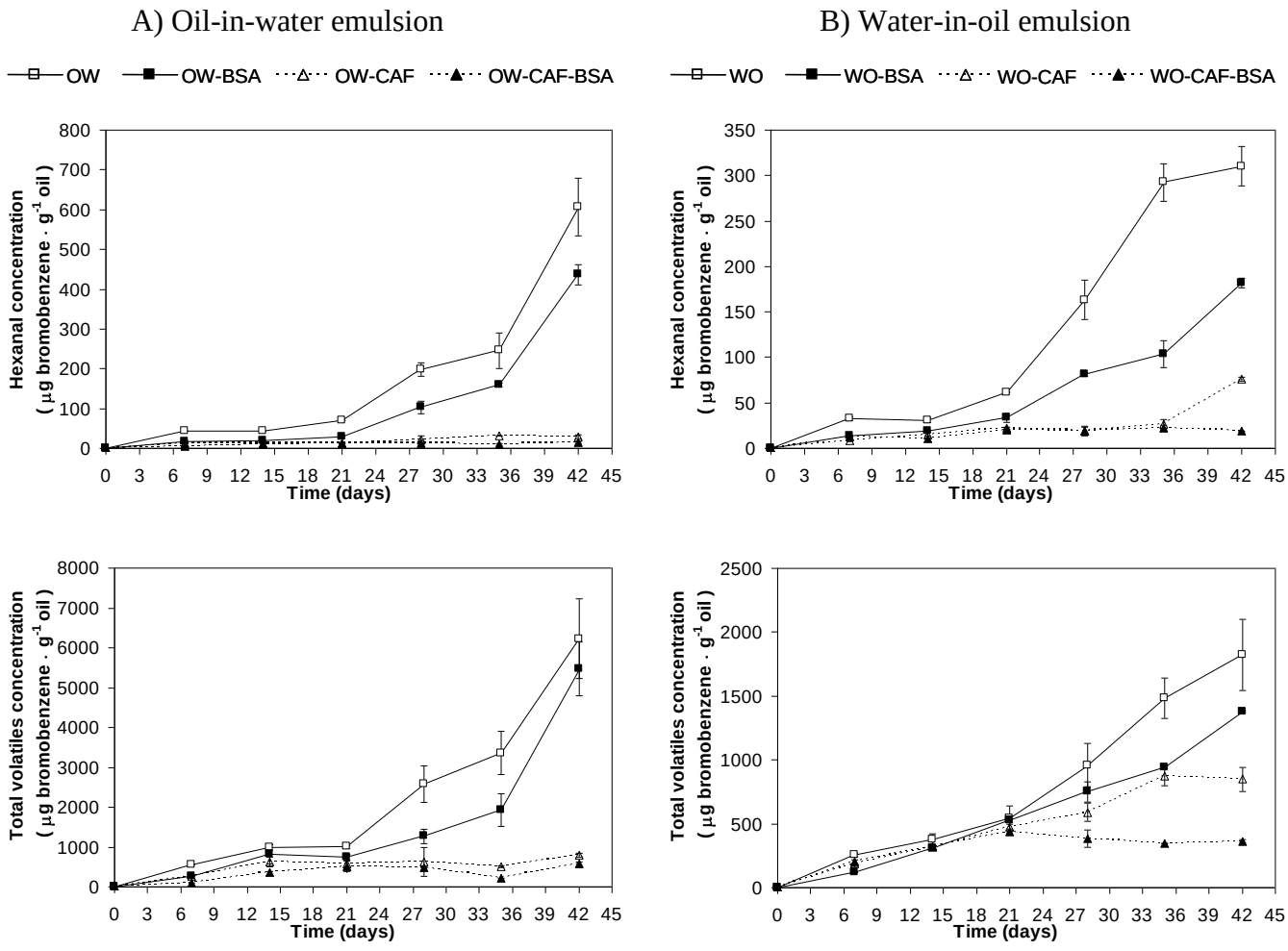


Figure 2. Changes in hexanal and total volatiles concentration of a) OW emulsions and b) WO emulsions containing antioxidants during storage at 50 ºC, with concentration expressed as area equivalent to bromobenzene.

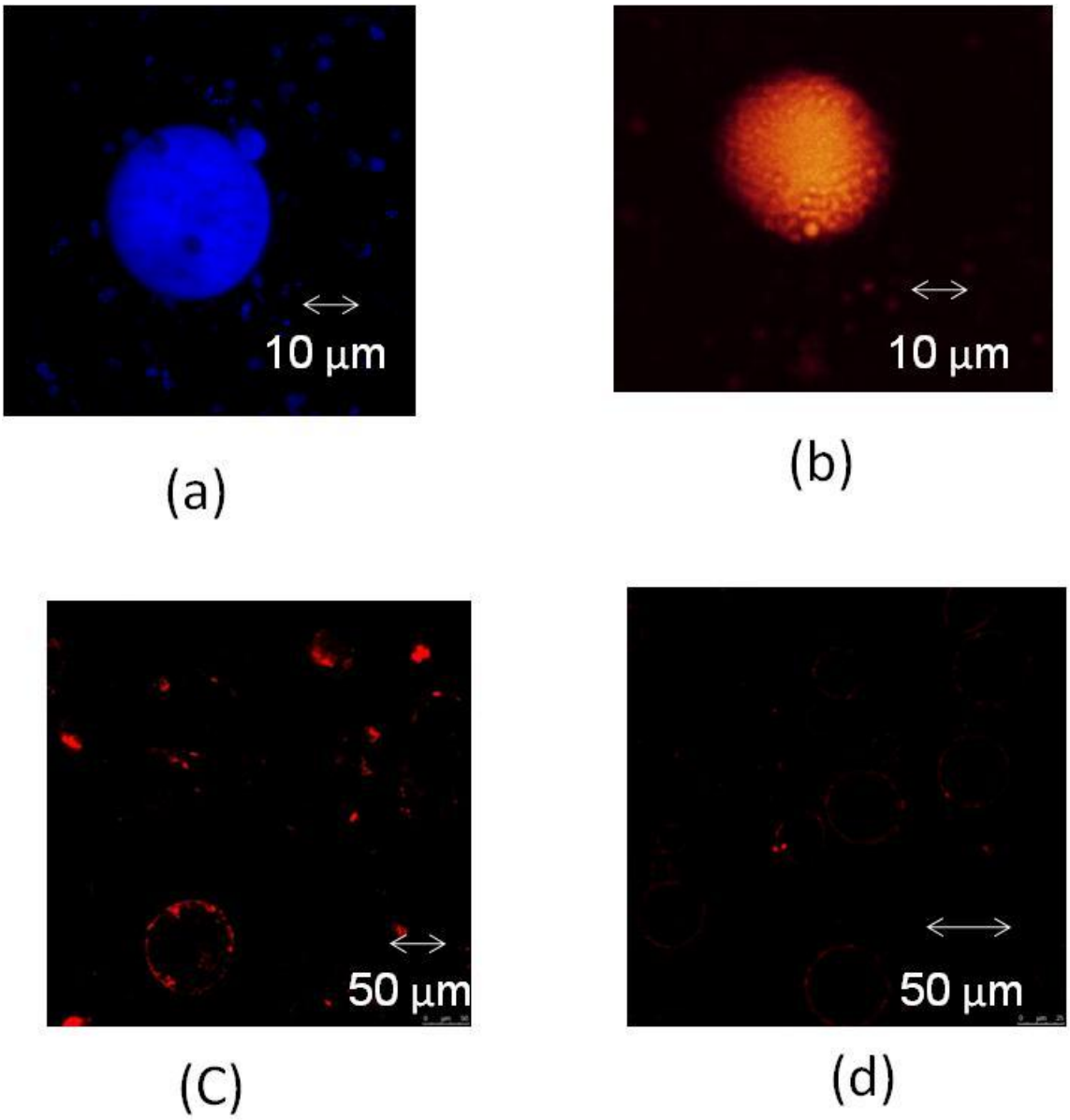
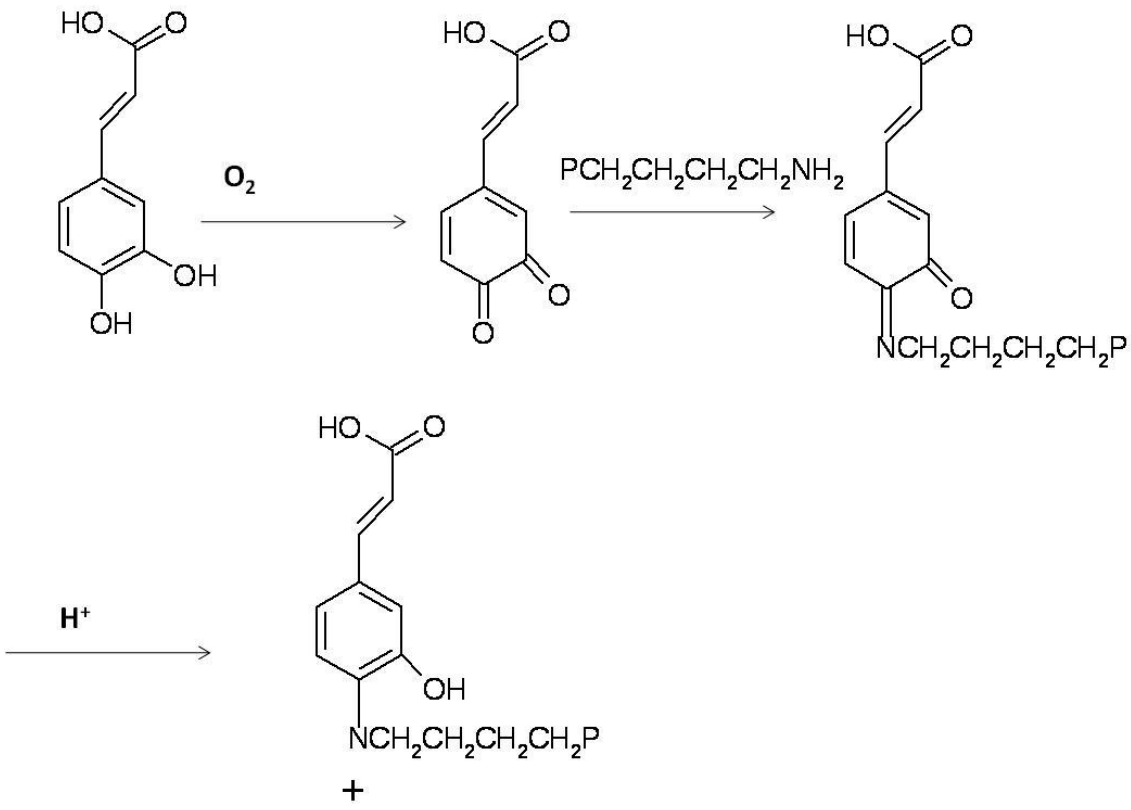


Figure 3. Emulsions stained with oil red- nile blue mixture (a) Oil-in-water emulsion with BSA; (b) Oil-in-water emulsion with no protein; (c) water-in-oil emulsion with BSA; (d) water-in-oil emulsion with no protein



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534 **Scheme 1**. Reaction of caffeic acid with lysine residue of BSA (P = protein backbone)