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Bovine Serum Albumin Produces a Synergistic Increase in the Antioxidant Activity of Virgin Olive Oil Phenolic Compounds in Oil-in-Water Emulsions

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Virgin olive oil is valued for its flavor, but unacceptable off-flavors may develop on storage in food products containing this oil due to oxidation. The oxidative stability of oil-in-water emulsions containing bovine serum albumin (BSA) and virgin olive oil phenolic compounds was studied. Four oil-in-water emulsions with and without BSA and phenols isolated from virgin olive oil were prepared. These model systems were stored at 60 °C to speed up lipid oxidation. Primary and secondary oxidation products were monitored every three days. Peroxide values and conjugated diene contents were determined as measures of the primary oxidation products. *p*-Anisidine values and volatile compounds were determined as measures of the secondary oxidation products. This latter determination was carried out by headspace solid-phase microextraction coupled with gas chromatography. Although olive oil phenolic compounds and BSA contributed some antioxidant activity when present as individual additives, the combination of BSA with phenols in an emulsion showed 58−127% synergy, depending on which analytical method was used in the calculation. The emulsion containing phenolic compounds and BSA showed a low level of deterioration after 45 days of storage at 60 °C.

KEYWORDS: Antioxidant; albumin; phenolic compounds; virgin olive oil; oxidative stability

INTRODUCTION

Fatty foods are commonly in emulsion form either as water-in-oil, for example, butter and margarine, or oil-in-water, for example, mayonnaise, milk and cream. Lipid oxidation leading to rancidity is often the decisive factor determining the useful storage life of food products, even when their fat content is very low as in some emulsions. Antioxidants are substances that, when present at low concentrations compared to those of an oxidizable substrate, significantly delay or prevent oxidation of that substrate (*1*), and olives and olive-derived products are recognized as a valuable source of natural phenolic antioxidants (*2, 3*). Olive oil hydrophilic extracts contain a large number of phenolic compounds including simple phenols, lignans, and secoiridoids (*4–6*), which exhibit antioxidant properties (*3, 7, 8*).

The activity of different types of antioxidants can vary significantly depending on whether the lipids are triacylglycerols, methyl esters, free fatty acids or are incorporated into various biological particles such as lipoproteins or liver microsomes. The location of antioxidants in aqueous, bulk lipid or in heterophasic systems has an important effect on their activity. The oxidative stability of most colloidal, lipid-bearing foods is greatly affected by a multitude of surface-active substances and their interfacial interactions with both oxidants and antioxidants (*9*). Antioxidant activity is strongly affected by the physical composition of the test system, partly due to partitioning of the antioxidants between the phases being important (*10*), and the relative activity of antioxidants of different polarity varies significantly in different multiphase systems (*11*). The observation that polar antioxidants are more active in bulk oil systems whereas nonpolar antioxidants are more active in lipid suspended in aqueous systems was referred to as the "polar paradox" by Porter (*11*). The effects of combinations of antioxidants also vary in lipid systems depending on the phases present (*12*).

Proteins have been shown to have weak antioxidant activity including both metal chelating and radical scavenging activity (*13*). However, the main activity of bovine serum albumin (BSA) in oil-in-water emulsions is its action in enhancing the antioxidant effect of water-soluble phenolic compounds (*14*). Strong synergistic increases in antioxidant activity were ob-

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served between BSA and catechins, caffeic acid and Trolox, but synergy was less between BSA and *tert*-butyl hydroquinone.

The aim of this study was to determine the influence of BSA on the total antioxidant activity of virgin olive oil phenols in a model food emulsion. The flavor of virgin olive oil is highly valued and the oil is very stable, but even for this oil rancid, oxidized flavor notes may develop on storage, and methods of retarding the development of these off-flavors are of interest. Understanding about interactions between phenolic components and albumin may also be relevant to their transport and properties in human physiology, where olive oil phenols may bind to albumin (*15*).

MATERIALS AND METHODS

Chemicals. The standard 3,4-dihydroxyphenylacetic acid (3,4-DHPAA) and the reagents used for quantification of phenols by HPLC were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). All solvents used were analytical or HPLC grade (Merck & Co. Inc., Darmstadt, Germany). Isooctane, glacial acetic acid, ferrous sulfate, barium chloride, potassium thiocyanate, polyoxyethylene sorbitan monolaurate (Tween-20), *tert*-butyl hydroquinone, sodium hydroxide, hydrochloric acid, sodium chloride, sodium sulfate, *p*-anisidine, cumene hydroperoxide, bovine serum albumin (BSA), hexanal, heptanal, octanal, nonanal, 2-pentylfuran, *E*-2-decenal, *E,E*-2,4-decadienal, *E*-2-undecenal and bromobenzene were purchased from Sigma-Aldrich. Extra virgin olive oil (EVOO) was purchased from a local retail outlet.

Extraction of the Phenolic Fraction. The phenolic fraction was extracted from the EVOO by a liquid/liquid extraction method, according to Pirisi et al. (*16*). The dry extracts were redissolved in 0.5 mL of a methanol/water (50:50, v:v) solution. Before being injected into the HPLC, the samples were filtered through 0.2 μm nylon filters (Whatman Inc., Clifton, NJ, USA).

Chromatographic Analysis by HPLC-DAD/MSD. HPLC analysis was carried out using a HP 1100 Series instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump delivery system, degasser, autosampler, diode array UV−vis detector (DAD), mass selective detector (MSD), in reverse phase using a C_{18} Luna column 5 μm, 25 cm × 0 mm ID (Phenomenex, Torrance, CA, USA) according to Rotondi et al. (*17*). Each phenolic compound identified was quantified as mg 3,4-DHPAA kg^{-1} oil (calibration curve with $r^2 = 0.9739$).

Removal of Phenols from Extra Virgin Olive Oil (EVOO). Phenolic compounds were extracted from EVOO (8 × 35 g) by liquid/liquid extraction with sodium hydroxide solution (0.5 M, 4 × 15 mL). After each extraction, the mixture was centrifuged at 1000 × *g* for 5 min and the aqueous phase was discarded. Combined olive oil fractions were then washed with hydrochloric acid solution (0.5 M, 2 × 10 mL) and with saturated sodium chloride solution (5 × 10 mL), centrifuged at 1000 × *g* for 5 min, dried with anhydrous sodium sulfate and filtered under vacuum. Dried olive oil (200 g) free of phenolic compounds was obtained.

Emulsion Preparation. Oil-in-water emulsions (30% oil, 4 × 2 × 125 g) were prepared by dissolving Tween-20 (1%) in acetate buffer (pH 5.4), either with or without BSA (0.2% w/w). Emulsions were prepared by the dropwise addition of EVOO (with or without phenols) to the water phase, cooling in an ice bath with continuous sonication by a Vibracell sonicator (Sonics and Materials, Newton, CT, USA) for 5 min. The emulsion samples were coded EV (without phenols and without BSA), EVA (without phenols but with BSA), EVP (with phenols but without BSA) and EVPA (with phenols and BSA).

Emulsion Oxidation. All emulsions were stored in duplicate in 250 mL glass bottles in the dark (inside the oven) at 60 °C. Two aliquots of each emulsion (2 × 2.5 g) were removed periodically for peroxide value (PV), conjugated diene content (CD), *p*-anisidine value (PA), pH value (pH) and analyses of volatile oxidation products by gas chromatography (GC).

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 × *g*, the clear upper layer (0.2 mL) was collected, and peroxides were quantified using a method based on that of Diaz et al. (*18*). Lipid peroxide concentration was determined using a cumene hydroperoxide standard curve ($r^2 = 0.9986$).

Spectrophotometric Determination of Conjugated Diene Content (CD) and *p*-Anisidine Value (PA). Prior to CD and PA analyses, oil was separated from the emulsion sample by freezing, thawing and centrifugation. The use of a saturated saline solution added before thawing improved separation. CD and PA analyses were determined by AOCS official Methods no. Ti 1a-64 and no. cd 18-90, respectively (*19, 20*).

Solid Phase Microextraction (SPME) Sampling Conditions. An aliquot of emulsion (1.96 ± 0.02 g) was weighed in a 20 mL vial. A magnetic follower was added and the vial was capped with a Teflon-faced rubber septum and plastic cap, before storing at −20 °C prior to analysis. The vial was placed in a water bath on a magnetic stirrer and the sample was equilibrated for 2 min at 60 °C. The septum was manually pierced with the SPME needle and the fiber was exposed to the emulsion headspace for 60 min and transferred to the gas chromatograph where the volatiles were desorbed in the injection port. The desorption time in the injection port was 15 min.

SPME/GC Analysis. Volatile oxidation compounds (hexanal, heptanal, octanal, nonanal, 2-pentylfuran, *E*-2-decenal, *E,E*-2,4-decadienal, *E*-2-undecenal) were monitored by headspace analysis with solid phase microextraction (HS-SPME) (*21*). A manual SPME fiber holder unit and 30 μm DVB-CAR-PDMS fiber (Sigma-Aldrich Company Ltd., Dorset, UK) were used to adsorb volatiles from the emulsion in a closed vial at 60 °C with a sampling time of 60 min. GC analyses were performed with a HP 5890 series II gas chromatograph (Agilent UK, South Queensferry, UK) equipped with FID detector and split/splitless injector. Chromatographic separation was carried out using a HP-5 column (15 m length, 0.25 mm ID and 0.25 μm film thickness; Agilent UK). The oven temperature was 40 °C for 10 min, followed by temperature programming to 140 at 2.5 °C min^{-1}, and then increased to 300 at 20 °C min^{-1}. Helium was used as carrier gas in the splitless mode. The FID temperature was 280 °C and the injection port was held at 260 °C. The identification of all compounds was based on the mass spectra determined by GC-MS using a HP 5890 series II gas chromatograph with MS detector and by comparison of their retention time with those of authentic standards.

Calculation of Synergy. Synergy was calculated as described in Almajano and Gordon (*14*).

$$\%\ \text{synergy} = 100\{[\text{IP(antioxidant + protein)} - \text{IP(control)}] - \frac{[(\text{IPantioxidant} - \text{IPcontrol}) + (\text{IPprotein} - \text{IPcontrol})]\}}{[(\text{IPantioxidant} - \text{IPcontrol}) + (\text{IPprotein} - \text{IPcontrol})]}$$

where IP = induction period.

Statistical Analysis. Chemical data were analyzed using Statistica 6.0 (Statsoft Inc., Tulsa, OK, USA). The significance of differences between means at a 5% level was determined by one-way ANOVA, using Tukey's HSD posthoc test. To verify the association among experimental data, Pearson correlation analysis was performed using the same statistical package; *p*-values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Evaluation of Phenolic Profile by HPLC-DAD/MSD. The EVOO and the same oil stripped of phenolic compounds, as previously described, were analyzed by HPLC-DAD/MSD to quantify the individual phenols. Phenolic compounds, classified as phenylethanol derivatives (hydroxytyrosol, HYTY, and tyrosol, TY), secoiridoids (decarboxymethyl oleuropein aglycone, DOA, oleuropein aglycone, OA, ligstroside aglycone, LA, oleocanthal, OL) and lignans ((+)-pinoresinol PIN and acetylpinoresinol, AcPIN) were identified and quantified. The total concentration of phenolic compounds was 41.93 ± 3.01 mg·kg^{-1}

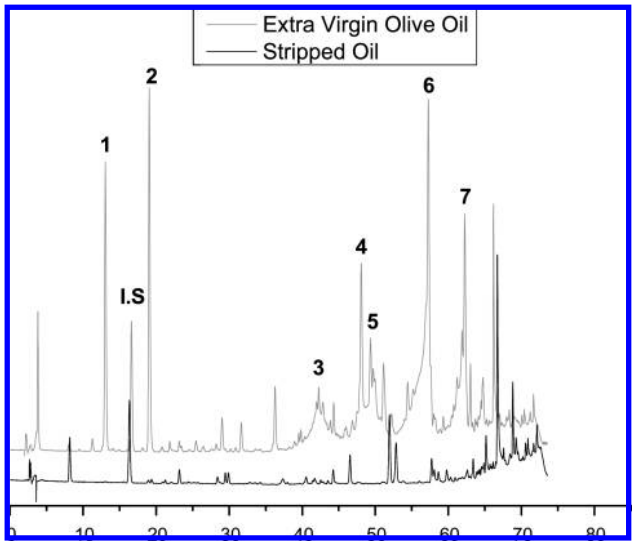

Figure 1. Phenolic profiles of the extra virgin olive oil and of the same oil, stripped of phenolic fraction. 1, hydroxytyrosol (HYTY); I.S., internal standard; 2, tyrosol (TY); 3, decarboxymethyl oleuropein aglycone (DOA); 4, (+)-pinoresinol (PIN); 5, (+)-1-acetoxypinoresinol + oleocanthal (AcPIN+OL); 6, oleuropein aglycone (OA); 7, ligstroside aglycone (LA).

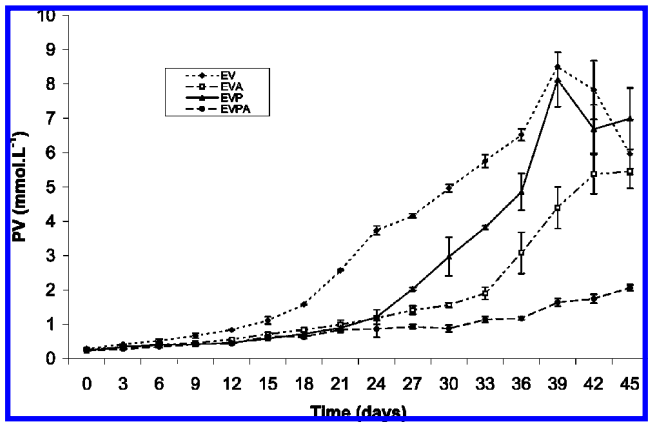

Figure 2. Changes in peroxide value of emulsion containing antioxidant during storage at 60 °C. EV, emulsion without phenols and albumin; EVA, emulsion without phenols but with albumin; EVP, emulsion with phenols but without albumin; EVPA, emulsion with phenols and albumin.

oil. The stripping of phenolic compounds from the oil by alkaline washing reduced the concentration of these compounds to a low level and as illustrated in **Figure 1** the stripped oil contained a very low content of phenols to confirm that stripping of phenols was effective. The decrease was between 82.9%, for DOA, and 100% for HYTY, PIN and OA. This stripping allowed the preparation of an oil phase without phenols used to prepare the emulsions EVA (with only BSA) and EV (without any additive).

Effect of BSA on the Antioxidant Activity of Olive Oil Phenols in Oil-in-Water Emulsion. The emulsions were stored at 60 °C to accelerate changes that should also be observed, but more slowly, at lower temperatures. Rancidity develops under these conditions by oxidation and not by lipolysis. The initial PV of the emulsion samples was similar with values between 0.22 mmol L^{-1} cumene hydroperoxide for EVPA and 0.28 mmol L^{-1} for EV. After 27 days of oxidation the four emulsions reached significantly different PV values in the order: EV > EVP > EVA > EVPA. As shown in **Figure 2**, the maximum PV was 8.50 mmol L^{-1} cumene hydroperoxide at

Table 1. Times in Days[a] for Oil-in-Water Emulsions Stored at 60 °C to Reach PV = 2.00 mmol L^{-1} Hydroperoxide, CD = 0.5%, Hexanal = 5 μg of Bromobenzene g^{-1} Oil and Total Volatiles = 10 μg of Bromobenzene g^{-1} Oil

emulsion[b]	PV[a]	CD[a]	hexanal[a]	total volatiles[a]
EV	21.7	17.9	21.0	14.6
EVA	30.5	26.1	30.9	24.0
EVP	27.3	21.8	26.2	18.3
EVPA	44.5	39.1	>45	44.3
% synergy	58	75		127

[a] Times calculated from data for duplicate samples fitted to an exponential equation. [b] EV, emulsion without phenols and albumin; EVA, emulsion without phenols but with albumin; EVP, emulsion with phenols but without albumin; EVPA, emulsion with phenols and albumin.

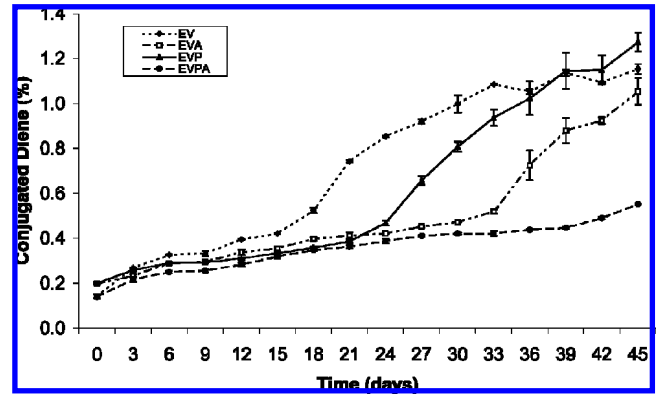

Figure 3. Changes in conjugated diene content of emulsion containing antioxidant during storage at 60 °C. EV, emulsion without phenols and albumin; EVA, emulsion without phenols but with albumin; EVP, emulsion with phenols but without albumin; EVPA, emulsion with phenols and albumin.

time point 13 (39 days) for EV, followed by 8.12 mmol L^{-1} cumene hydroperoxide for EVP; then there was a decrease in PV for these samples as the rate of primary oxidation compound formation became less than primary oxidation compound decomposition as observed previously (*22*). As shown in **Table 1**, EV took 21.7 days to reach PV = 2.00 mmol L^{-1} cumene hydroperoxide, followed by EVP with 27.3 days, then EVA with 30.5 days and finally EVPA, the most stable, with 44.5 days.

The same trend is highlighted in **Figure 3**, for CD determination. A positive correlation between CD and PV ($r = 0.96$, $p < 0.05$) was found as shown in **Table 2**. EVPA emulsion presented the highest time value to reach CD = 0.5%: in fact, that was 39.1 days compared with the 26.1 days for EVA, 21.8 days for EVP, and 17.9 days for EV. The values of CD content became significantly different after 12 days.

The PA value determination, a measure of secondary oxidation products (*22–24*), was in agreement with the PV analysis. A good positive correlation (**Table 2**) was found between PA and PV ($r = 0.96$, $p < 0.05$) and also between PA and CD ($r = 0.95$, $p < 0.05$). At time zero EVP showed the highest PA value with 2.89 and EV, EVA and EVPA were not significantly different from each other. At time 9, after 27 days, EV had the highest value, followed by EVP, EVA and EVPA. These four values were significantly different ($p < 0.5$). At time 15, after 45 days of oxidation, the differences between the four emulsions were higher with EV at 82.72, EVP at 73.50, EVA at 55.35 and EVPA, the emulsion with albumin and phenols, at 7.87 (**Figure 4**.)

Table 3 shows PV, CD and PA data of the emulsions at time 15 (after 45 days of oxidation).

Table 2. Significant Pearson's Correlations ($p < 0.05$) among Parameters Analyzed[a]

Pearson's correlation:	PV	CD	PA	hexanal	heptanal	2-pentylfuran	octanal	nonanal	*E*-2-decenal	*E,E*-2,4-decadienal	*E*-2-undecenal	Total volatiles
PV	–	0.96	0.96	0.86	0.80	0.78	0.85	0.89	0.92	0.92	0.91	0.92
CD	0.96	–	0.95	0.84	0.78	0.77	0.83	0.90	0.90	0.93	0.88	0.90
PA	0.96	0.95	–	0.92	0.89	0.88	0.93	0.95	0.96	0.90	0.94	0.97
hexanal	0.86	0.84	0.92	–	0.84	0.87	0.90	0.88	0.87	0.79	0.85	0.94
heptanal	0.80	0.78	0.89	0.84	-	0.96	0.97	0.94	0.93	0.74	0.92	0.94
2-pentylfuran	0.78	0.77	0.88	0.87	0.96	-	0.95	0.92	0.89	0.70	0.86	0.92
octanal	0.85	0.83	0.93	0.90	0.97	0.95	–	0.97	0.95	0.78	0.94	0.97
nonanal	0.89	0.90	0.95	0.88	0.94	0.92	0.97	–	0.98	0.86	0.96	0.98
E-2-decenal	0.92	0.90	0.96	0.87	0.93	0.89	0.95	0.98	–	0.89	0.99	0.98
E,E-2,4-decadienal	0.92	0.93	0.90	0.79	0.74	0.70	0.78	0.86	0.89	–	0.88	0.88
E-2-undecenal	0.91	0.88	0.94	0.85	0.92	0.86	0.94	0.96	0.99	0.88	–	0.97
total volatiles	0.92	0.90	0.97	0.94	0.94	0.92	0.97	0.98	0.98	0.88	0.97	–

[a] PV, peroxide value; CD, conjugated diene content; PA, *p*-anisidine value.

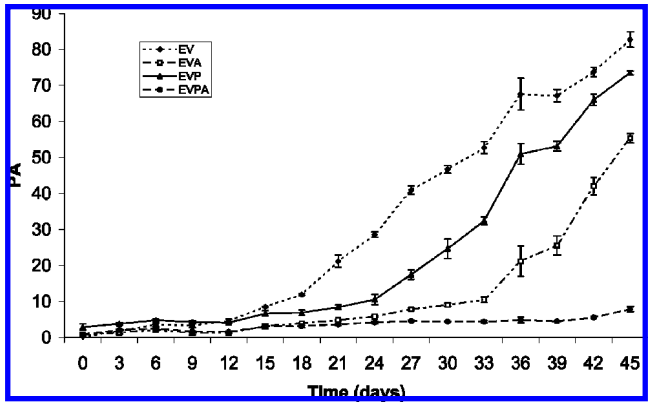

Figure 4. Changes in *p*-anisidine value of emulsion containing antioxidant during storage at 60 °C. EV, emulsion without phenols and albumin; EVA, emulsion without phenols but with albumin; EVP, emulsion with phenols but without albumin; EVPA, emulsion with phenols and albumin.

Table 3. Data, after 45 days of Oxidation, Expressed as Mean ± Standard Deviations of Four Determinations[a]

	EV	EVA	EVP	EVPA
PV	$5.96 \pm 1.00^{a,b}$	5.45 ± 0.09^{b}	6.99 ± 0.90^{a}	2.06 ± 0.09^{c}
CD	1.15 ± 0.02^{b}	1.05 ± 0.06^{c}	1.27 ± 0.04^{a}	0.55 ± 0.00^{d}
PA	82.72 ± 2.12^{a}	55.35 ± 1.27^{c}	73.50 ± 0.54^{b}	7.87 ± 0.70^{d}

[a] Same letters within each row do not significantly differ ($p < 0.05$). EV, emulsion without phenols and albumin; EVA, emulsion without phenols but with albumin; EVP, emulsion with phenols but without albumin; EVPA, emulsion with phenols and albumin. PV, peroxide value; CD, conjugated diene content; PA, *p*-anisidine value.

HS-SPME analysis was carried out to monitor individual volatile secondary oxidation products. Headspace analysis by SPME is a suitable method for evaluating the degree of oxidation of virgin olive oils (*21*). Most volatile compounds are formed by autoxidation, except hexanal, which is formed both by autoxidation and by the lipoxygenase pathway. Heptanal, octanal, nonanal, 2-pentylfuran, *E*-2-decenal, *E,E*-2,4-decadienal and *E*-2-undecenal can be considered as markers of the degree of oxidation, although some of them do not have a particularly significant impact on flavor due to their high odor thresholds (*25, 26*).

Hexanal is the main volatile formed during the oxidation of lipids via linoleic acid 13-hydroperoxide (*25, 26*). At time 0 the hexanal peaks were equal in area to values between 0.07 and 0.24 μg bromobenzene g^{-1} oil for all emulsions and were not significantly different,. The time to reach a hexanal peak area equal to 5 μg of bromobenzene g^{-1} oil was 21.0 days for EV, 26.2 days for EVP and 30.9 days for EVA. The EVPA emulsion did not reach this limit during the experiment (**Table 1**).

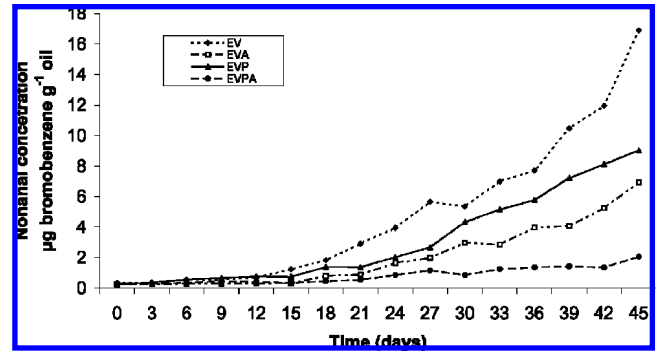

Figure 5. Changes in nonanal content of emulsion containing antioxidant during storage at 60 °C, with concentration expressed as area equivalent to bromobenzene. EV, emulsion without phenols and albumin; EVA, emulsion without phenols but with albumin; EVP, emulsion with phenols but without albumin; EVPA, emulsion with phenols and albumin.

Nonanal is another major volatile formed during oxidation of an emulsion containing oil rich in oleic acid (*18, 24*). Its formation in the emulsions was in the same order as hexanal with EV > EVP > EVA > EVPA as illustrated in **Figure 5**. After 15 days the nonanal peak areas corresponded to 16.90, 9.03, 6.93 and 2.07 μg bromobenzene g^{-1} oil respectively.

Among the volatile oxidation products analyzed by HS-SPME, the lowest rates of formation were observed for 2-pentylfuran, which is consistent with the findings of Vichi et al. (*21*). The relative formation of 2-pentylfuran in the emulsions (**Figure 6**) was in the same order as hexanal and after 45 days the peak area values corresponded to EV = 7.50, EVP = 5.17, EVA = 3.85, EVPA = 0.65 μg bromobenzene g^{-1} oil. The formation of all other individual volatiles (heptanal, octanal, *E*-2-decenal, *E,E*-2,4-decadienal and *E*-2-undecenal) (**Table 2**) showed a similar relative order of formation, being formed fastest in EV, followed by EVP, EVA and EVPA. Once the volatiles were all detected consistently after day 9, the ratio of volatile concentrations for all emulsions was reasonably consistent with hexanal, *E*-2-decenal, *E*-2-undecenal, nonanal, *E,E*-2,4-decadienal, octanal, heptanal and 2-pentylfuran representing an average of approximately 24%, 23%, 20%, 12%, 8%, 6%, 4% and 2% of the total volatiles.

Figure 7 shows the development of total volatile oxidation products in the emulsions. To reach a total volatile peak area corresponding to 10 μg of bromobenzene g^{-1} oil, EV took 14.6 days, EVP 18.3 days, EVA 24.0 days and EVPA 44.3 days (**Table 1**).

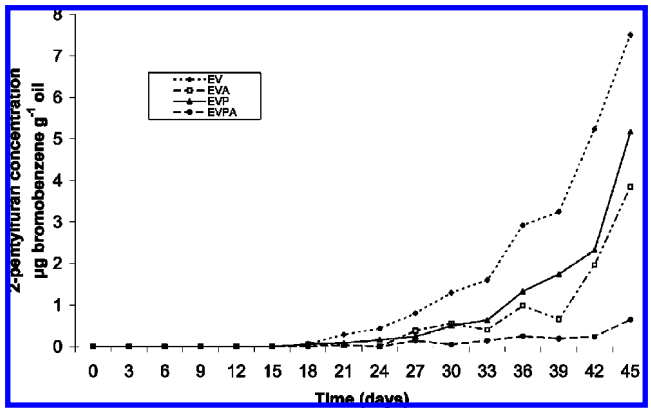

Figure 6. Changes in 2-pentylfuran content of emulsion containing antioxidant during storage at 60 °C, with concentration expressed as area equivalent to bromobenzene concentration. EV, emulsion without phenols and albumin; EVA, emulsion without phenols but with albumin; EVP, emulsion with phenols but without albumin; EVPA, emulsion with phenols and albumin.

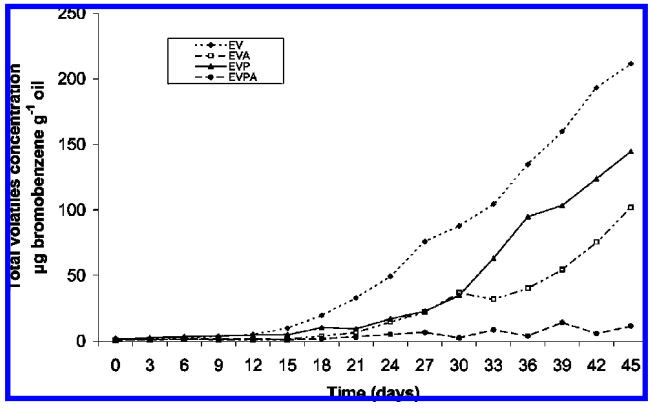

Figure 7. Changes in content of total volatile oxidation compounds of emulsion containing antioxidant during storage at 60 °C, with concentration expressed as area equivalent to bromobenzene concentration. EV, emulsion without phenols and albumin; EVA, emulsion without phenols but with albumin; EVP, emulsion with phenols but without albumin; EVPA, emulsion with phenols and albumin.

In conclusion, the order of stability of the four oil-in-water emulsions was EVPA > EVA > EVP > EV, which was confirmed by all the analyses. In other words, the emulsion containing phenols and BSA was much more stable than those containing only BSA or phenols or neither additive. The % synergy can be calculated as 58% based on PV measurements, 75% based on CD measurements or 127% based on total volatiles (**Table 1**). It can be concluded that BSA exerts a synergistic effect with the phenolic antioxidants and the hypothesis has been proposed by Almajano et al. (*14*) that this is due to formation of a protein-antioxidant adduct during storage. It is suggested that the protein-antioxidant adduct is concentrated at the oil−water interface due to the surface-active nature of the protein. Phenolic compounds can bind irreversibly to protein on storage, according to Almajano and Gordon (*28*), and the effect in an oil-in-water emulsion is a synergistic increase in antioxidant activity if both protein and phenols are present. This work indicates that proteins may play a useful role in retaining optimal flavor of food emulsions containing virgin olive oil.

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