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Physical and Oxidative Stability of Functional olive Oil-in-Water Emulsions Formulated Using Olive Mill Wastewater and Whey Proteins

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Physical and oxidative stability of functional olive oil-in-water emulsions formulated using olive mill wastewater and whey proteins

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ABSTRACT

The olive oil extraction process implies the production of high amounts of wastewater, rich in phenolic compounds, derivatives of oleuropein. The present paper reports on the use of these extracts added in model olive oil-in-water (O/W) emulsions to study their effects on physical and chemical stability. Spray-dried polyphenols extracted from olive mill wastewater (OMWW) were added to a model 20% olive O/W emulsion stabilized with whey protein isolate (WPI) and xanthan gum, in phosphate buffer solution at pH 7. The emulsions were characterised over accelerated storage conditions (40 °C) up to 30 days. Physical stability was evaluated by analysing the creaming rate, mean particle size distribution and mean droplet size, viscosity and rheological properties, while chemical stability was assessed through the measurement of primary and secondary oxidation products. The rheological behaviour and creaming stability of the emulsions was dramatically influenced by xanthan gum, whereas the concentration of WPI and the addition of encapsulated OMWW phenolics did not result in a significant improvement of physical stability. Interestingly, the formation of oxidation products was higher when higher concentrations of encapsulated polyphenols were used, indicating a possible binding with WPI added in the system as natural emulsifier.

***Keywords*:** O/W emulsions, olive oil phenolic compounds, whey protein isolate, emulsion rheology, oxidation, functional food.

Abbreviations: OMWW: olive mill waste water; WPI: whey protein isolate; O/W: oin-in-water; Ty: tyrosol; OHTy: hydroxytyrosol; PV: peroxide value; TBARS: thiobarbituric acid derivative species. Abbreviations used for the emulsion systems: HX, high xanthan (0.2%); LX: low xanthan (0.06%); HP: high WPI (0.5%); LP: low WPI (0.13%).

1. Introduction

Olive oil production technology implies major environmental problems in the countries where its production is mainly localized, i.e. the Mediterranean area, as industry produces a high output of liquid by-products, represented by the olive mill wastewater (OMWW). Due to the high concentration of phenolic compounds (Servili et al., 1999), this waste could be conveniently converted into a valuable source of antioxidant compounds, which can be added to a variety of foods to develop a functional product with better nutritional properties (Obied et al., 2005).

In recent few years, new technologies have been tested and applied for the extraction of phenolic compounds from wastewater, particularly membrane processes which implies ultrafiltration in combination with nanofiltration and reverse osmosis (Paraskeva & Diamadopoulos, 2006). The concepts behind its production by membrane separation techniques are reported by other researchers (Akdemir & Ozer, 2009; Gkoutsidis et al., 2011). For their convenient storage and use, water phenolic extracts must be dried, and spray-drying has been applied to OMWW obtained from membrane filtration. Their use in O/W emulsions, is interesting and applicable at industrial level for the production of a wide range of functional food products, like mayonnaise, creams, sauces and other spreads.

Emulsions are kinetically unstable systems, and their instability is due to many mechanisms, including creaming, coalescence and flocculation (Dickinson, 2009; McClements, 2004). Therefore, stabilizers and emulsifiers are needed to provide physical stability to avoid the natural separation phase. Food emulsions are often multiphase systems containing more than one biopolymer, e.g. mixtures of proteins and polysaccharides (Moschakis, Murray, & Biliaderis, 2010). Thickening agents are mainly polysaccharides, e.g. xanthan gum, maltodextrin, galactomannans, starches, pectin, carboxymethycellulose, etc., used to increase viscosity of the continuous phase (Dickinson, 2009; Paraskevopoulou, Boskou, & Kiosseoglou, 2005).

Milk proteins (caseinate and whey proteins) are hydrocolloids used in many food systems, for their good solubility and behaviour under heating conditions (Huck-Iriart, Álvarez-Cerimedo, Candal, & Herrera, 2011). Being surface active, whey protein isolates (WPI) are adsorbed on the oil–water interface in the form of a protective film (Djordjevic, McClements, & Decker, 2004; Sun & Gunasekaran, 2009; Sun, Gunasekaran, & Richards, 2007). Proteins are usually less effective emulsifiers than synthetic surfactants, but their use in food industry has being increasing due to the trend to use “clean label” ingredients or “natural” products.

Many factors have been considered for the characterisation of O/W emulsions, including the effect of pH, oil phase concentration (Dapčević Hadnađev, Dokić, Krstonošić, & Hadnađev, 2013), the composition of the oil phase used,the effect of stabilizers, emulsifiers, droplet size and droplet size distribution (Lethuaut, Métro, & Genot, 2002; Silva, Rocha-Leão, & Coelho, 2010), and the presence of metals and phenolic compounds (Paiva-Martins & Gordon, 2002; Sørensen et al., 2008). The effect of environmental stresses, such as heating, chilling, freezing and drying, was also reported, as it influences the behaviour of protein-stabilized emulsions (McClements, 2004).

Olive oil has been studied by various researchers to formulate O/W emulsions (Bylaite, Nylander, Venskutonis, & Jönsson, 2001; Protonotariou, Evageliou, Yanniotis, & Mandala, 2013), e.g. in mixture with lemon juice to simulate a traditional Mediterranean dressing, which was stabilized using propylene glycol alginate, xanthan or gum Arabic (Paraskevopoulou et al., 2005). Olive oil was shown to have better physical stability in low-fat emulsion, stabilized by WPI and xanthan, than other oils like sesame oil (Protonotariou et al., 2013). Previous works also reported on the characterisation of model O/W emulsions (Di Mattia, Sacchetti, Mastrocola, & Pittia, 2009; Di Mattia, Sacchetti, Mastrocola, Sarker, & Pittia, 2010; Sotiroudis, Sotiroudis, Varkas, & Xenakis, 2005).

The complexity of real emulsions is due to the presence of mixtures of proteins and polysaccharides, and in such systems the addition of phenolic compounds implies further possible interactions, particularly with proteins, which lead to complex coarcervation between

macromolecules. This latter phenomenon is largely affected by ionic strength, biopolymer ratio, biopolymer concentration and by their distribution and charge density (Moschakis et al., 2010). In single proteins, e.g. β-lactoglobulin, BSA etc., the effect of polyphenols binding was previously reported (Almajano & Gordon, 2004). In emulsions, the presence of the aqueous phase can decrease the activity of antioxidant compounds (Kumamoto, Sonda, Nagayama, & Tabata, 2001). Hydroxytyrosol, the most abundant phenolic compound in olive oil and OMWW, was reported to exert the highest antioxidant activity among olive phenolics (Paiva-Martins & Gordon, 2002). The antioxidant activity of more polar phenolic compounds was reported to be reduced in emulsions compared to bulk oil, for the so-called "polar paradox". Previous studies shown that antioxidant activity of pure hydroxytyrosol, oleuropein and its derivatives varies according to the pH conditions (Paiva-Martins & Gordon, 2002).

There is a lack of information about the behaviour of biopolymers in olive O/W emulsions and their effect on emulsion stability, as the effect of phenolic compounds depends upon many factors (Frankel & Meyer, 2000). Olive oil is a complex mixture of minor compounds and triacylglycerols, better stabilisation was attributed to the interaction with minor compounds and whey proteins (Di Mattia et al., 2010).

The Food industry has the need to verify these effects on real food products and promising benefits are expected from the application of phenolic extracts from OMWW, both in terms of environmental and nutritional value of foods. Indeed, the conversion of by-products into valuable ingredients for the design of functional food products has been investigated using olive oil as a source of fat, whey protein isolate and olive phenolics in a model O/W emulsion system.

Therefore, the aim of this paper was to characterise the behaviour and properties of O/W emulsions formulated with 20% olive oil and functionalised by adding polyphenols extracts from OMWW powder extract at two concentrations, stabilized by WPI and xanthan gum.

2. Material and Methods

2.1. *Olive oil sample, stabilizers and OMWW powder*

Freshly refined olive oil was donated by I.O.B.M. srl (Montesarchio, BN, Italy). Xanthan gum from *Xanthomonas campestris* was purchased from Sigma-Aldrich (Darmstadt, Germany). WPI was 97.5 wt% protein, and lactose content was less than 1 wt%. A phosphate buffer solution at pH 7.0 was prepared using monosodium phosphate and sodium hydroxide (Darmstadt, Germany). The buffer was used to maintain constant pH, as this parameter can affect emulsion stability (Sørensen et al., 2008). All other chemicals were of analytical grade purity. Phenolic extract from olive mill wastewater (OMWW) was kindly donated by LABS (Department of Agriculture, University of Naples Federico II, Italy). OMWW production process has been reported by Troise et al. (2014).The composition of the three main phenolic compounds analysed by UPLC-UV-Vis was as follows: OHTy 32±0.2 mg g^{-1}, Ty 1.9±0.1 mg g^{-1}, verbascoside 2.8±0.09 mg g^{-1} (Troise et al., 2014).

2.2. Emulsions preparation

Emulsions were prepared by dispersing different amounts of spray-dried OMWW powder (1-5 mM expressed as OHTy) and WPI (0.13 or 0.5% w/v) into a buffer solution (5mM phosphate buffer, pH 7). The aqueous phase was gently stirred for 2 h at room temperature to ensure dissolution, using a magnetic stirring bar and magnetic stirrer hotplate (Stuart CB162, Bibby-scientific, Staffordshire, UK). The pH was checked and adjusted to pH 7.0 using 1M HCl. Xanthan gum (0.06 or 0.2% w/v) was added to the emulsions and gently stirred (100 rpm) overnight at room temperature to allow complete hydration. Emulsions were produced by blending 20% (v/v) refined olive oil in the solution previously prepared using a high-speed blender at 8'000 rpm for 2 min, after a pre-emulsification phase (Traynor, Burke, Frias, Gaston, & Barry-Ryan, 2013). The emulsions

were then transferred in glass tubes for the creaming stability analysis, and stored into an incubator at 40 °C ±1 °C for kinetic stability evaluation (**Fig. 1**).

The concentrations of the stabilisers were chosen according to previous works (Di Mattia et al., 2009; Sun et al., 2007). Emulsions with low or high concentrations of stabilisers are named as follows: LX-LP, low xanthan (0.06% w/v) and low WPI (0.13% w/v); LX-HP, low xanthan and high WPI (0.5% w/v); HX-LP, high xanthan (0.2% w/v) and low WPI; HX-HP, high xanthan and high WPI. For each system, two concentrations of OMWW extract were used, i.e. 1 and 5 mM as OHTy, corresponding to low and high average biophenols concentrations in commercial virgin olive oils (Caporaso et al., 2015). Blank system had no added OMWW. In this case, WPI and xanthan was 0.5% and 0.2% w/v, respectively.

2.3. Creaming value

Creaming value was monitored visually according to literature (Dickinson, Radford, & Golding, 2003). Duplicate samples of emulsions were stored in 75 mm x 12 mm sample tubes (York Glassware, UK) at 25 ±0.5 °C. Measurement of the serum layer (creaming index) was carried out manually using a 60% fiberglass Measy 2000 Typ 5921 (Baty, Switzerland). Stability was evaluated as percentage decrease from the initial height, using the following formula: Creaming Index=100 × (H_S/H_E); where H_S is serum layer formed at the bottom of glass tubes, and H_E is the total height of the emulsions in the tubes (Klinkesorn, Sophanodora, Chinachoti, & McClements, 2004).

2.4. Mean particle size by image analysis (optical microscopy)

For particle size determination through digital image analysis, emulsions were diluted 1:1000 using buffer solution to avoid droplet overlapping. A drop of emulsion was placed on a microscope

slide and then covered with a cover slip. The microstructure of the emulsion was observed using an Olympus DP72 optical microscopy (Japan) at 40x and 400x magnification. Digital pictures were taken by using an Olympus E-620 digital camera mounted on the microscope. Mean droplet size was calculated by analysing the microscopic images with ImageJ 1.47t 64-bit software (National Institutes of Health, USA). The function Analyse Particles was used after colour threshold and using the following options: size 0.01-infinity; circularity: 0.00-1.00; exclude on edges; particle size >2 μm^2; options exclude droplets on edges and include holes. At least 20 pictures were taken at 100x magnification for each emulsion analysed. Image analysis was performed as reported by previous works (Silva et al., 2010), where details of the method were reported.

2.5. Emulsion droplet size distribution

Droplet-size distributions of the emulsions were determined by using a Mastersizer 2000 Hydro 2000S (Malvern Instruments, UK), which gives measurement based on light scattering under high dilution conditions by dispersing the samples in distilled water (Dickinson et al., 2003; Lethuaut et al., 2002). To avoid multiple scattering effects, the freshly prepared emulsions were diluted to reach an obscuration rate of about 3. The refractive indices of water and refined olive oil were 1.330 and 1.418, respectively. Average droplet sizes were characterized in terms of the volume mean diameter $d_{4,3} = \Sigma_i \cdot n_i \cdot d_i^4 / \Sigma_i \cdot n_i \cdot d_i^3$, where n_i is the number of droplets and of diameter d_i. The $d_{4,3}$ parameter is a useful mean diameter value, sensitive to small changes in droplet-size distribution (Moschakis et al., 2010). All measurements were made at room temperature and four measurements were obtained for each sample. A bimodal particle-size distribution was taken to be indicative of non-reversible flocculation (Dickinson et al., 2003; Lethuaut et al., 2002).

2.6. Cloudiness and turbidity measurements

Cloudiness measurement, also called turbidity or opacity, was carried out according to previously published methods for O/W emulsions (Mirhosseini et al., 2008). Samples were taken after 24 h to their preparation and analysed over the storage period. In the case of separated emulsions, the supernatant was sampled, while in the absence of evident phase separation, the upper part was sampled as well. Emulsions were diluted (2.5:1000) and cloudiness was expressed from the absorbance at 660 nm.

2.7. Rheological properties of emulsions

Rheological measurements were carried out in accordance to previous papers reporting on O/W emulsions stabilized by WPI and xanthan gum (Sun & Gunasekaran, 2009). Steady shear viscosity and small-amplitude oscillatory shear tests were conducted using a Bohlin C-VOR dynamic rheometer (Malvern Instruments Inc., Southborough, MA). Emulsion viscosity was measured at 25°C, over a shear rate range of 0.01–100 s^{-1} with cone-plate geometry (CP 40/4°). All measurements were performed within 24 h from emulsion preparation. A logarithmic progression was applied, and sweep time was 120 s. Oscillatory tests were performed by pouring emulsion samples (typically 1-1.5 mL) directly on the holding stage and samples were covered with thin paraffin oil layer preventing water evaporation. In oscillatory experiments the storage (G') and loss (G'') moduli were recorded versus frequency (0.1–10 Hz) at constant strain, with increasing of logarithmic scale. The linear viscoelastic region was previously determined selecting a strain of 0.5 Pa, recording G' and G'' versus shear stress (0.01–100 Pa) at constant frequency.

2.8. Oxidative stability

2.8.1. Lipid hydroperoxides

Lipid hydroperoxides were measured according to literature (Di Mattia et al., 2009). Emulsions (0.3 mL) were mixed with 1.5 mL of isooctane/2-propanol (2:1, v/v), vortexing three times for 30 s and centrifuging for 2 min at 2000 x g (Hettich Rotanta 460R centrifuge). The supernatant (200 μL) was collected and 2.8 mL of a methanol:1-buthanol solution (3:1, v/v) were added, followed by 15 μL of 3.94 M ammonium thiocyanate and 15 μL ferrous iron solution (prepared by adding equal amounts of 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). After 20 min, absorbance was measured at 510 nm using a Lambda Bio 20 spectrophotometer (Perkin Elmer, Boston, MA). Hydroperoxides concentration was determined using a calibration curve prepared with hydrogen peroxide.

2.8.2. Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARs) were determined according to previously published methods (Di Mattia et al., 2009; Di Mattia et al., 2010). Emulsions (0.1-1 mL) were mixed with 2.0 mL of TBA reagent (15% w/v tricholoracetic acid and 0.375% w/v thiobarbituric acid in 0.25 M HCl) in test tubes and placed in a boiling water bath for 15 min. The tubes were cooled to room temperature for 10 minutes and then centrifuged (2000 g using a Hettich Rotanta 460R centrifuge) for 15 min at 20 °C. After 10 minutes, the absorbance was measured at 532 nm. TBARs concentration was determined by a standard curve prepared using 1,1,3,3-tetramethoxypropane.

2.9. Statistical analysis

In order to better understand the influence of polyphenols, WPI and xantan, as well as their interactions, a multifactor ANOVA with second-order interactions was performed. XLStat (2009.3.02), add-in software package for Microsoft Excel (Addinsoft Corp., Paris, France), was used for data elaboration.

3. Results and discussion

3.1 Creaming rate

The creaming rate of olive O/W emulsions added with OMWW and stabilized by WPI and xanthan gum is reported in **Fig. 2**. Different behaviour was observed depending on xanthan gum levels. This hydrocolloid is a stabilizer, it was expected to delay creaming when using higher concentrations. Indeed, xanthan is widely applied for its good viscoelastic and chemical properties, e.g. water solubility and pH stability (McClements, 2004). The control sample contained a medium concentration of xanthan (0.2% w/v) and the creaming was lower in the first storage period, i.e. up to 8 days, where a separation similar to the samples containing low concentration was reached.

The addition of OMWW did not cause dramatic changes in the creaming rate between low and high concentrations of xanthan gum. In the systems with low OMWW level (**Fig. 2a**), a difference in the creaming rate was observed due to the concentrations of WPI. While in the emulsions with low xanthan concentration a statistical difference was not reached, samples with high xanthan concentration showed higher creaming rate when higher WPI levels was used.

Two different creaming processes exist, i.e. the creaming of individual particle, with particle size around 10 μm, and the migration of flocculates, which is observed at lower particle sizes (Huck-Iriart et al., 2011). Creaming occurs when the density of the droplets is less than that of the continuous phase (Dickinson & Golding, 1997), and stabilizers act in increasing emulsion viscosity and therefore they retard droplets movement.

Protein-polysaccharide conjugates are known to be formed in WPI-stabilized emulsions with polysaccharides, which lead to improved physical stability. Our results about emulsion's physical stability are in agreement with previous literature data particularly for the stabilising effect of xanthan gum on the creaming rate (Krstonošić, Dokić, Dokić, & Dapčević, 2009; Traynor et al.,

2013). However, excessive xanthan gum might induce higher creaming rate by depletion flocculation, as droplet flocculation influences creaming stability of monodisperse oil-in-water emulsions (Chanamai & McClements, 2001). Creaming rate in case of droplet flocculation is generally higher at increased particle size and lower with droplet concentration, due to hydrodynamic effects and particle–particle interactions (Chanamai & McClements, 2001).

A possible effect of WPI on creaming rate was reported by Sun and Gunasekoran (2009), as increasing WPI concentrations caused a slight decrease in creaming index. Indeed, it has been reported that 0.2% of WPI was not enough to cover the entire droplet surface to stabilize the emulsions containing 20 to 40% oil (Sun & Gunasekaran, 2009). The effect was explained by the unabsorbed WPI in the aqueous phase, while the presence of xanthan increased the amount of protein unabsorbed at the interface. The different behaviour observed in our emulsions with low OMWW and high concentration of xanthan (HX-LP and HX-HP) is likely to be due to this phenomenon. The possible protein-polyphenols interaction seems to be minimal and explained by the chemical structure of olive phenolics and by the presence of other compounds that could interfere with the binding, particularly xanthan, which might represent a barrier for the physical interaction and consequently the chemical binding. Indeed, OMWW phenolics have a relatively simple structure and lower molecular weight than other classes of phenolics for which the protein-polyphenols effect was reported, i.e. tannins.

Previous papers have shown that olive phenolics have binding affinity with caseinate and whey proteins, whereas OHTy and Ty did not interact significantly with pure BSA (Pripp, Vreeker, & van Duynhoven, 2005). In our previous study (Genovese, Caporaso, De Luca, Paduano, & Sacchi, 2015) the effect of the interaction between olive phenolics and WPI on headspace release of volatile compounds in emulsion was reported. Particularly, a significant increase of some key odour compounds was found when phenolic compounds were added to the emulsion, it was hypothesised that the polyphenol−protein interaction influenced the binding of volatile compounds by WPI.

Trying to better understand our results, a multifactorial ANOVA analysis was carried out. The influence of polyphenols, WPI, xanthan, and their interactions, on the analysed parameters over storage time were evaluated (**Table 1**). The results showed that creaming is apparently influenced by all variables and by interactions between polyphenols-WPI and polyphenol-xanthan. Moreover, the statistical analysis shown that storage influenced all the other parameters considered. For creaming rate, a significant effect of the interaction between OMWW and WPI was found, as well as OMWW and xanthan.

3.2 Mean droplet size by image analysis and droplet size distribution

Droplet size distribution in freshly-prepared emulsions was assessed by measuring both $d_{3,2}$ and $d_{4,3}$ using a Mastersizer (**Fig. 3**). It was reported that $d_{4,3}$ is more sensitive to the presence of large particles in emulsions than $d_{3,2}$, therefore it is often more sensitive to phenomenon such as coalescence (McClements, 2004). A bimodal distribution of particle size was observed, which also implies a high standard deviation of the average particle size.

Particle size in O/W emulsions was expected to be influenced by WPI level (Yuan, Gao, Decker, & McClements, 2013), as higher WPI concentrations were reported to cause lower particle size diameter (Sun & Gunasekaran, 2009). However, the authors applied a high pressure homogenizer to reach a considerable lower mean particle size, while in our case the absence of dramatic differences are likely to be due to the lower exposed surface area, as the diameter of the particle size in our samples was circa 10 times higher. The authors explained the lower particle size was due to a larger area stabilized by the higher amount of added protein and to a faster coverage of the droplet surface (Yuan et al., 2013).

In our emulsions, $d_{3,2}$ was influenced by high xanthan concentration (**Fig. 3a**), as at higher levels a lower average particle size was measured, probably due to the effect of this hydrocolloid on the stabilization after a few hours from emulsion production. Some emulsifier agents have been

reported to have no significant effect on the mean droplet diameter, but xanthan gum was reported to be a critical factor (Hemar, Tamehana, Munro, & Singh, 2001). The effect of xanthan gum on droplet size of emulsions is controversial, as some papers indicated a decrease in mean droplet diameter at increased xanthan concentration (Krstonošić et al., 2009), while others reported an increase in droplet size attributed to the flocculation caused by the hydrocolloid (Hemar et al., 2001; Ye, Anema, & Singh, 2004). Others reported that droplet size of O/W emulsions was unaffected by the presence of xanthan in the range 0-0.15% (w/v) (Moschakis, Murray, & Dickinson, 2005). A more obvious difference was observed between the systems containing low and high OMWW concentration, with generally higher $d_{4,3}$ in the second case, with the only exception of LX-HP (**Fig. 3b**). This suggests a possible destabilisation effect of olive oil phenolics, which caused greater droplet dimensions. Our results of $d_{3,2}$ are in accordance with previous papers that report an increase in xanthan concentration leads to decrease in droplet average diameter (Krstonošić et al., 2009).

The change in mean droplet size was also assessed by digital image analysis of micrographs, over storage (**Fig. 4**). A general increasing trend in the particle diameter was measured up to 23 days of storage, particularly evident at the last period of incubation. This information could appear in contrast with the creaming observation, as the main changes were observed during the first week, while a plateau was observed later. Previous reports suggest that there could be an increase in droplet size without any direct effect on creaming rate when emulsions are stabilized by thickening agents (Reference???). The mean droplet sizes assessed by image analysis were bigger than the value obtained by light scattering technique, i.e. Mastersizer analysis. This effect was explained by the impossibility of detecting the smallest droplets in the micrographs. This aspect was also reported previously by other authors (Moschakis et al., 2005). From our micrographs, it was observed that the main driver affecting the structure of the O/W emulsions was xanthan, as the presence of some aggregates was observed at high xanthan gum concentrations (data not shown), in accordance with previous findings (Moschakis et al., 2005).

No dramatic influence of OMWW extract on mean droplet size was observed, except at the last sampling time. At day 23, droplet size increased in the presence of phenolics by a factor of two as compared to the control. It was noted, moreover, that in samples with high xanthan concentration, the particle size was significantly ($P<0.05$) higher than in emulsions with lower levels. Xanthan gum level had statistically significant effect on droplet size. The interaction of phenolics during time, and polyphenols with WPI was significant (**Table 1**). Xanthan gum effect is explained by the way in which this compound stabilises emulsions, i.e. acting as a thickener and therefore retarding the phase separation, whereas they do not act directly in the maintenance of small particle size diameters. As widely known, both the presence of insufficient emulsifier and its excess in the aqueous solution could lead to different droplet size of the obtained emulsions (McClements, 2004).

Previous literature reported a general increase in droplet size during accelerated storage of olive O/W emulsions, also depending on the type of phenolic compounds used, and their possible interference on protein rearrangements was shown (Di Mattia et al., 2010). However, in our case, OMWW contains phenolics which are less reactive toward protein-protein interaction and therefore a more limited effect was expected.

3.3 Cloudiness

Cloudiness of the emulsions under accelerated conditions showed a general decreasing trend over storage time (**Fig. 5**). Cloudiness is dependent upon the type and concentrations of hydrocolloids, and the variation in its value obtained in our experiment is in accordance with previous findings (Mirhosseini et al., 2008). It was explained by the changes in average droplet size induced by the aggregation of oil droplets as well as the changes in the refractive index of oil phase and aqueous phase. Turbidity loss was explained by mechanisms such flocculation, coalescence and aggregation, which are responsible for the turbidity loss over storage (Mirhosseini et al., 2008). Higher level of xanthan caused higher cloudiness values especially during the first day of storage.

OMWW resulted in significant effects on emulsion cloudiness (**Table 1**), as the measured value was significant lower when higher concentrations were added. At low OMWW concentration (**Fig. 5a**), higher levels of WPI caused higher cloudiness values, while the opposite effect was found at high OMWW concentrations (**a'**). This phenomenon could support the idea of protein-polyphenols interactions occurring in such a system as the hydroxyl group can interact with protein and in turn lead to a lower turbid emulsion. Statistically, the interaction of OMWW with both WPI or xanthan gum were significant (**Table 1**).

The general decrease in turbidity could be explained by emulsion phase separation. The serum and cream layers of the separated emulsions were separately analysed to verify this hypothesis, as shown in **Fig. 6**. The absorbance of serum was significantly higher than that of the cream layer, due to the higher presence of phenolic extract in the water phase, as these phenolic compounds are highly hydro-soluble. A possible influence of the xanthan gum concentration was also observed. This was expected due to the optical properties of the phenolic extracts, as it was suggested that linoleic acid oxidation affect the structural organisation of the micellar/emulsion system, leading to a turbidity increase (Sotiroudis et al., 2005).

3.4 Emulsions viscosity and rheological behaviour

As shown in **Fig. 7**, the most influencing factor for emulsion viscosity and rheological property was xanthan gum concentration. It caused a sharp increase in the measured viscosity. A significant effect wasfound for xanthan gum concentration on O/W emulsion viscosity, and the shear-thinning behaviour found in the samples and is in accordance with previous research(Moschakis et al., 2005; Sun et al., 2007). The presence of OMWW caused a further increase in viscosity than the control, probably due to the presence of maltodextrin as coating agent of the phenolic powder.

The viscoelastic region of the emulsions was determined by amplitude sweep test, and both levels of xanthan gum resulted in linear viscoelastic region (data not shown), in accordance with

others (Krstonošić et al., 2009). Small amplitude oscillatory shear measurement to define the oscillatory sweep properties of the emulsions was shown in **Fig. 8**. In all systems, the value of both moduli increased in the range 0.01-2 Hz (**Fig. 8a**). The storage and loss moduli (G'-G'') were unaffected by protein level, which is in accordance with previous findings (Sun & Gunasekaran, 2009). The only exception was represented by the emulsion with high OMWW level and high xanthan concentration. In this case, the addition of higher WPI concentration caused a significant decrease in shear stress. The increase in G' in time was attributed to the formation of strong droplet flocculation, and the gel-like rheological behaviour of protein-stabilized emulsions was attributed to the network structure formed by protein coating (Dickinson & Golding, 1997). As the viscous modulus (G'') was higher than the elastic one (G'), it indicates pseudo-plastic behaviour. The shear stress over the shear rate of the samples showed that xanthan gum was the main driving factor, while a significant difference was observed between low and high WPI concentrations at high OMWW level (**Fig. 8b**). This phenomenon is likely due to the weak interactions occurring among WPI and OMWW.

Oscillatory rheological measurements of storage modulus and loss modulus can indicate whether the emulsion system is strongly or weakly associated. Xanthan gum influenced emulsion yield stress, as low xanthan systems were obviously differentiated from those with high xanthan concentration, while both the presence of WPI and OMWW did not significantly influence emulsion rheological behaviour. Based on the theory of droplet flocculation, the viscoelasticity of the flocculated emulsions should increase with increasing xanthan gum concentration, whereas the thickening effect of this polysaccharide causes a delay of this phenomenon (Moschakis et al., 2005).

3.5 Lipid oxidation

The results for lipid oxidation test are reported in **Fig. 9**. An increase in lipid hydroperoxides concentration was observed up to circa 1 week upon storage in both systems (low and high

OMWW), with a limited extent in those with high WPI concentration (**Fig. 9a**). These results suggest a possible antioxidant effect of WPI. The concentration of primary oxidation products followed a stabilisation period in the final storage period. In this latter period, a slight increase was observed in the system with low OMMW, while in the high-OMWW one the oxidation products were lower than the control. Hydroperoxides concentration was dramatically lower in emulsions with phenolics, being LX-HP the only exception. However, a clear statistically significance was not reached for primary oxidation products depending on emulsion formulation, except for storage time (**Table 1**).

The results obtained for TBARS were quite different (**Fig. 9b**), as their concentration in systems with high OMWW was circa 3 fold higher than low OMWW. Also in this case, the higher peak concentration was obtained at about 1 week storage. Generally, high WPI concentration was associated with higher TBARS production, especially when olive phenolics were present at high concentration. In this latter case TBARS value was always higher than the control. WPI and xanthan significantly ($p<0.05$) influenced TBARS concentration, and the interaction of WPI with OMWW also resulted statistically significant (**Table 1**).

The higher TBARS concentrations are related to the decomposition of primary oxidation products, and therefore the formation of aldehydes and other products leading to off-flavour in the final product. In accordance with Di Mattia et al. (2010 and 2009), the presence of phenolic extracts in systems with low OMWW had only limited effects on lipid hydroperoxides formation. On the contrary, in high OMWW concentration, the oxidation level was higher in the system with high WPI (HX-HP). Accordingly, certain phenolic compounds, e.g. catechin, caused higher TBARS concentration and they are not able to delay the formation of primary oxidation products, showing pro-oxidant activity (Di Mattia et al., 2009; Di Mattia et al., 2010). Some phenolic compounds can exert the opposite effects on the chemical stability, e.g. caffeic acid retarded the production of peroxide while promoting at the same time higher concentration of secondary oxidation products (Sørensen et al., 2008). Zhou et al. (2013) confirmed the potential of some phenolic compounds to

act as antioxidants or pro-oxidants in O/W emulsions, being strongly influenced by the pH and their concentration. The authors reported that epigallocatechin-gallate had higher TBARS concentration (in the range pH 2-4) in the range 1-100 μM.Up to 500 μM, the observed effect was lower, and the authors explained by the competition between antioxidant and pro-oxidant effects (Zhou & Elias, 2013). Moreover, the possible presence of metallic ions as traces in our phenolic extract might have caused effects on the phenolic compounds and lipid oxidation. Metallic ions affect O/W emulsion oxidation and a pro-oxidant effect was reported for hydroxytyrosol and oleuropein in the presence of ferric ion, depending on the pH (Paiva-Martins & Gordon, 2002).

This demonstrates the importance of further studying lipid oxidation in O/W emulsions considering the effects of phenolics and proteins, as well as the presence of other stabilizers.

The interaction between olive oil phenolic compounds and food proteins was reported previously (Pripp et al., 2005). The authors studied sodium caseinate, bovine serum album, β-lactoglobulin and gelatin, in comparison to gallic acid and tannic acid. A relatively weak binding capacity was shown, as both OHTy and Ty had little or no binding. However, previous studies show that the amount of free phenols in the aqueous phase of emulsions decreases with increasing protein concentration, whereas the decrease was to a small extent in emulsions system than in aqueous solutions only (Pripp et al., 2005). Milk proteins have been reported as antioxidant compounds, e.g. casein hydrolysates and caseinophosphopeptides, being capable of binding transitional metals and limit lipid oxidation in O/W emulsions (Díaz, Dunn, McClements, & Decker, 2003). However, the effectiveness of WPI in emulsions was assessed not considering OMWW or olive phenolics in such systems (Sun & Gunasekaran, 2009).

Whereas OMWW seem not have positive effects on hydroperoxides or TBARS, their effectiveness *in vivo* is likely to be possible, as the polysaccharide coating could be broken during the digestion and therefore its phenolic content may be available to exert its antioxidant effects. Further work is needed in these aspects.

4. Conclusions

This paper reported on the stability of the model oil-in-water emulsions functionalized with polyphenols by addition of olive mill wastewater. It has been shown that emulsion stability, both from physical and chemical viewpoints, is dramatically dependent upon the type and concentration of the added stabilizers. The presence of OMWW could increase the creaming rate and physical instability, with a more limited influence on the rheological behaviour. At high concentrations of OMWW and WPI, both the physical and chemical stability was negatively affected, showing particularly high TBARS values. The addition of phenolic extracts is not straightforward as expected in terms of oxidative stability, and their interaction with the hydrocolloids, mainly whey proteins, can exert a significant influence and lead to different creaming rates, turbidity and rheological behaviours, but also have pro-oxidant effects at certain conditions. These results suggest the importance of accurately choosing the concentration of stabilizers, and it is suggested that lower WPI concentrations should be used when OMWW is added in food emulsions. The knowledge of olive O/W emulsion stability is of great importance for the formulation of emulsion based food products having enhanced health properties due to the widely known health benefit of this oil. Moreover, the possibility of using by-products from olive oil production process with high environmentally impact is of interest for food industry, as well as for food scientists to incorporate hydrophilic phenolic compounds in fatty foods. This research has great potential in terms of applicability at industrial level, and it is hoped to contribute to the hot topic of environmental pollution reduction and for the creation of innovative functional foods.

Further work is needed to better define the optimum concentrations of each hydrocolloid according to the final use of the product, as well as to understand the *in vivo* effect of the consumption of these functional emulsions.

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Figure captions

Figure 1. Olive O/W emulsions at day 7 of storage under accelerated storage (40 ºC). Olive O/W emulsions (20% fat content, v/v) were divided into two groups, with low and high concentration of added olive mill wastewater (OMMW) spray-dried phenolic extracts. Low (L) and high (H) concentrations of xanthan gum (X) and whey protein isolate (P) were used. Blank sample had no OMWW added, with 0.2% (w/v) xanthan gum and 0.5% (w/v) whey proteins.

Figure 2. Creaming stability of 20% (v/v) olive oil-in-water emulsions stabilized by WPI and xanthan gum, and added with low (**a**) of high (**a'**) concentration of OMWW, under accelerated storage conditions (40 ºC).

Figure 3. Mean droplet size, surface-weighted mean diameter ($d_{3,2}$) and volume-weighted mean diameter ($d_{4,3}$) in O/W emulsions with low (**a**) and high (**a'**) concentrations of OMWW, stabilized by xanthan gum (HX/LX) and whey protein isolate (HP/LP).

Figure 4. Mean droplet size analysed by digital image analysis over storage time, in samples added with OMWW at low (**a**) and high (**a'**) concentrations. Each data point represents the weighted average from at least 20 pictures containing 400-1800 droplets.

Figure 5. Cloudiness index measured at 660 nm in functionalised O/W emulsions with OMWW at low (**a**) and high (**a'**) concentrations.

Figure 6. Absorbance at 600 nm of the cream and serum layer of emulsions with low (**a**) and high (**a'**) OMWW levels, xanthan gum (HX/LX) and WPI (HP/LP) at day 7 of storage (40 ºC).

Figure 7. Shear-rate depence of the apparent viscosity of O/W emulsions with low (**a**) and high (**a'**) concentration of OMWW.

Figure 8. Viscous (G', filled) and elastic (G'', unfilled) moduli of emulsions at low (**a**) and high (**a'**) OMWW concentrations. Rheograms obtained on freshly prepared 20% olive O/W emulsions added with low (**b**) and high (**b'**) OMWW concentrations, at 25 ºC.

Figure 9. Lipid hydroperoxides (**a**) and secondary oxidation products TBARS (**b**) upon storage of emulsions. Phenolic compounds concentration was 150 (**a-b**) and 750 (**a'-b'**) mg kg^{-1} expressed as OHTy.

Table 1. Two-way ANOVA analysis with interactions considering the storage time, level of OMWW (polyphenols), WPI and xanthan gum, on different parameters analysed to assess emulsion stability.

	Creaming		**Droplet size**		**Cloudiness**		**Hydroperoxides**		**TBARS**	
Variable	F ratio	*p* value	F ratio	*p* value	F ratio	*p* value	F ratio	*p* value	F ratio	*p* value
Time	283.631	**< 0.0001**	224.42	**< 0.0001**	18.162	**< 0.0001**	46.710	**< 0.0001**	9.459	**0.002**
Polyphenol	105.837	**< 0.0001**	1.711	0.191	6.653	**0.012**	0.000	0.999	8.118	**0.005**
WPI	23.503	**< 0.0001**	0.728	0.394	6.080	**0.016**	0.049	0.825	0.094	0.760
Xantan	20.277	**< 0.0001**	8.822	**0.003**	0.815	0.370	0.132	0.717	4.907	**0.028**
Time*Polyphenol	0.140	0.708	5.154	**0.023**	5.462	**0.023**	0.208	0.649	1.851	0.175
Time*WPI	0.956	0.329	0.710	0.400	2.310	0.134	0.007	0.933	0.714	0.399
Time*Xantan	0.823	0.365	17.075	**< 0.0001**	0.218	0.642	0.002	0.964	3.286	0.072
Polyphenol*WPI	6620.019	**< 0.0001**	4.437	**0.035**	5.741	**0.020**	0.255	0.614	11.817	**0.001**
Polyphenol*Xantan	55.185	**< 0.0001**	2.695	0.101	39.212	**< 0.0001**	0.298	0.585	0.102	0.750
WPI*Xantan	2.570	0.110	2.278	0.132	1.069	0.305	0.989	0.321	2.813	0.095

In bold: significance of the explanatory variable (p <0.05)

Figure 1.

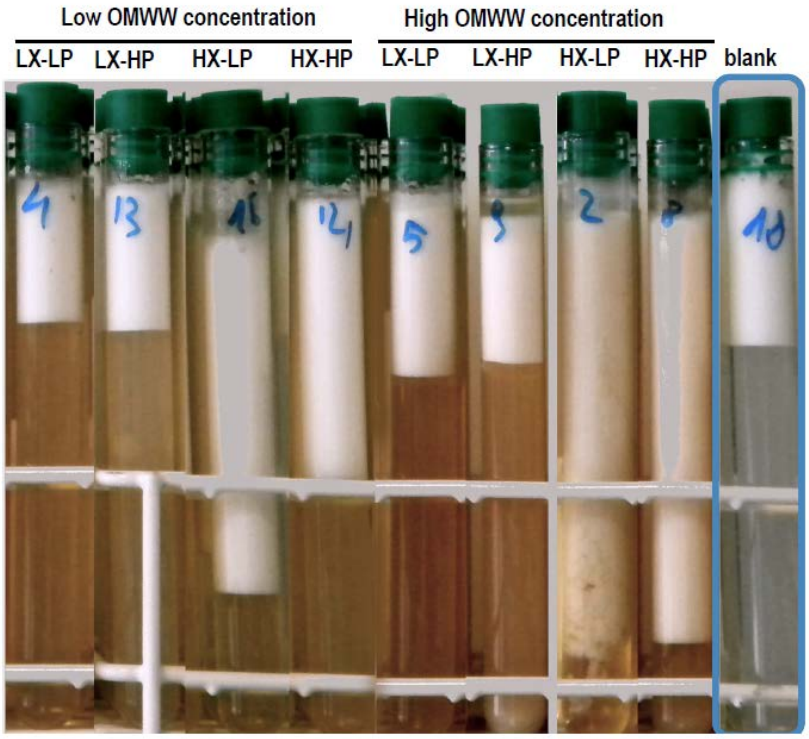

Low OMWW concentration
High OMWW concentration
LX-LP
LX-HP
HX-LP
HX-HP
LX-LP
LX-HP
HX-LP
HX-HP
blank

Figure 2.

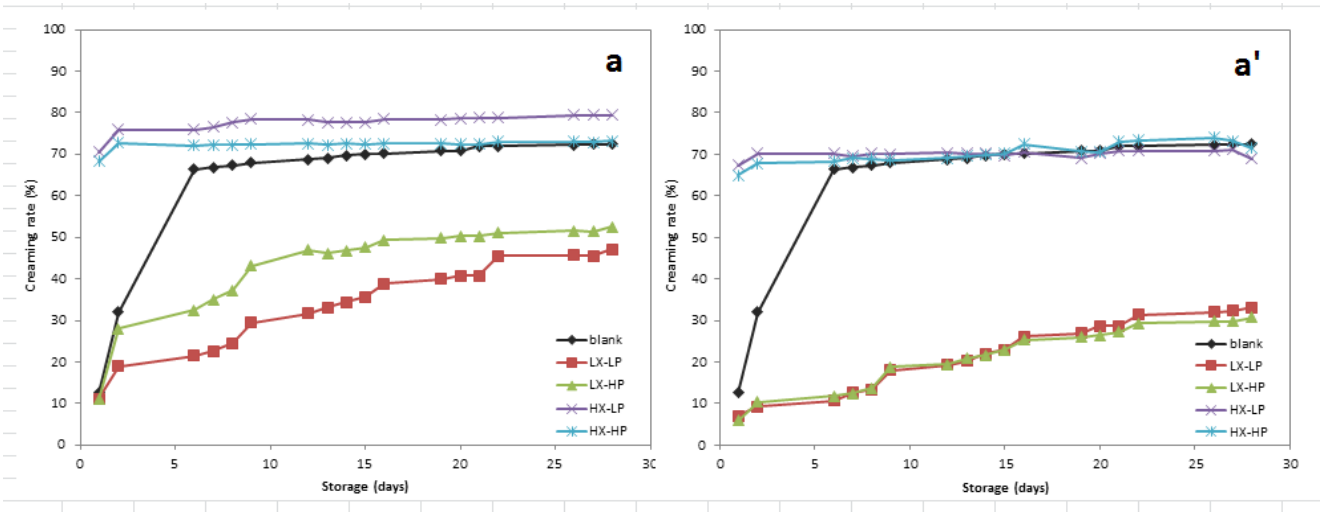


Figure 3.

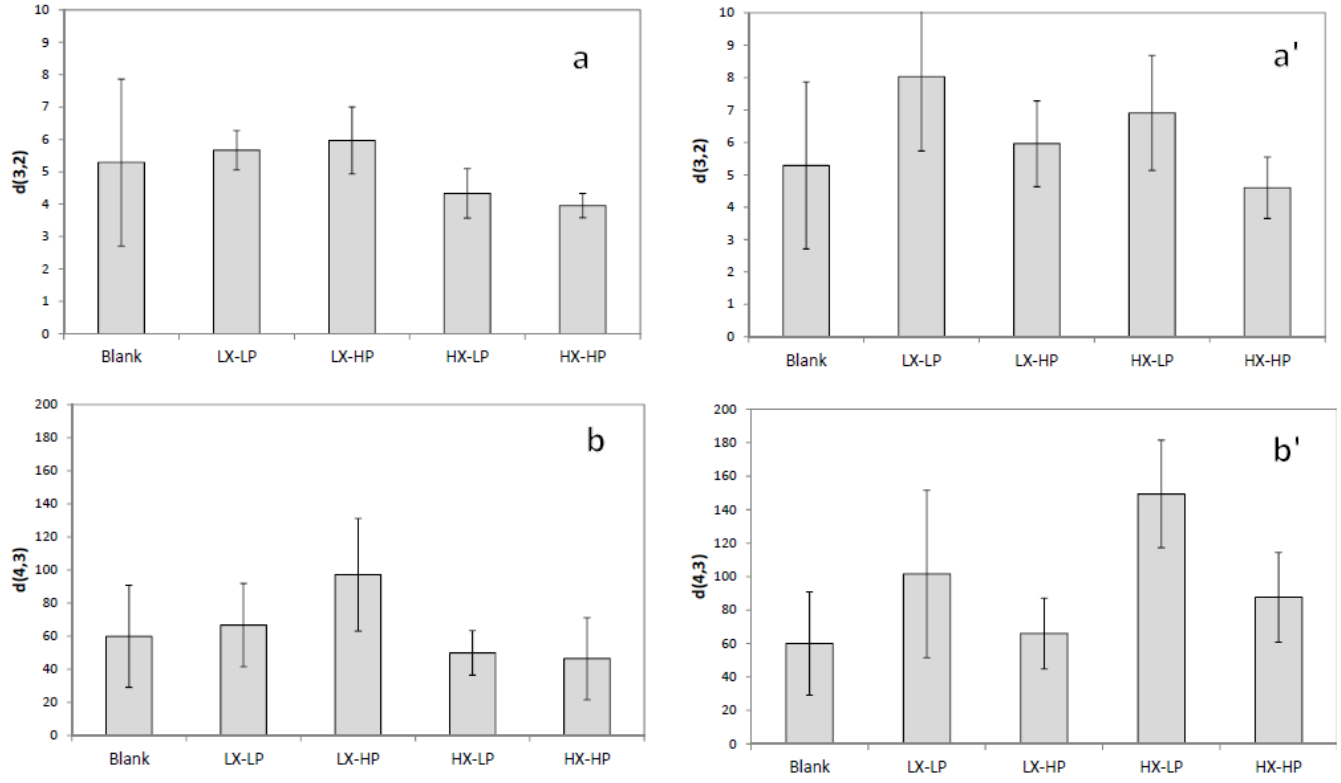


Figure 4.

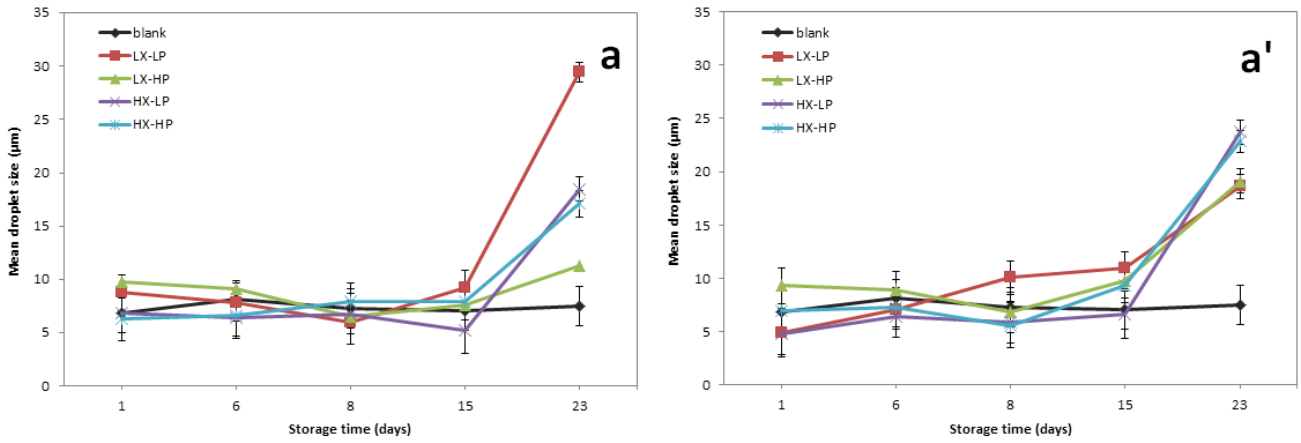

a
a'
blank
LX-LP
LX-HP
HX-LP
HX-HP
Mean droplet size (μm)
Storage time (days)
0
5
10
15
20
25
30
35
1
6
8
15
23

Figure 5.

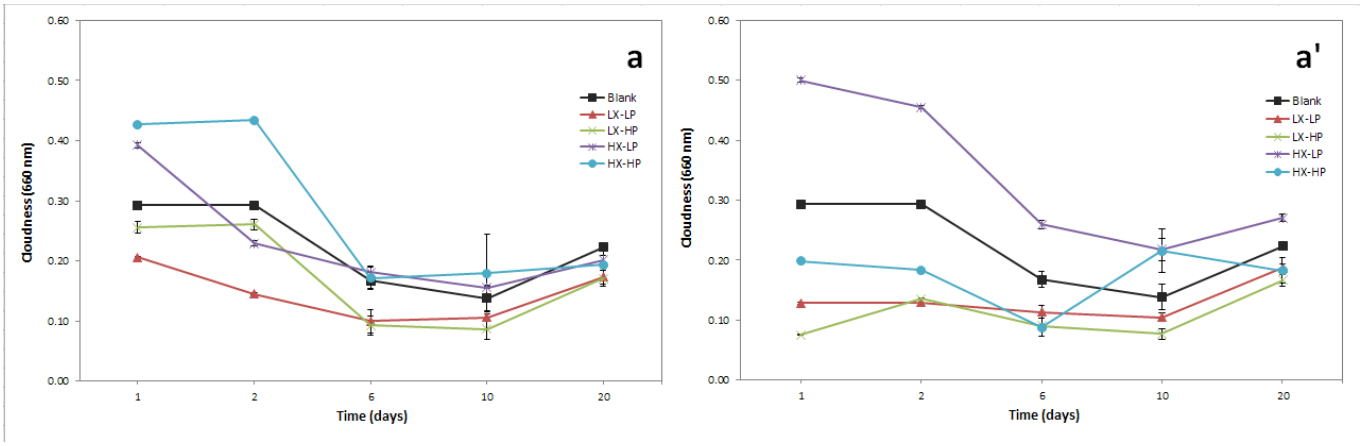

a
Cloudness (660 nm)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
1
2
6
10
20
Time (days)
Blank
LX-LP
LX-HP
HX-LP
HX-HP
a'
Cloudness (660 nm)
Time (days)
Blank
LX-LP
LX-HP
HX-LP
HX-HP

Figure 6.

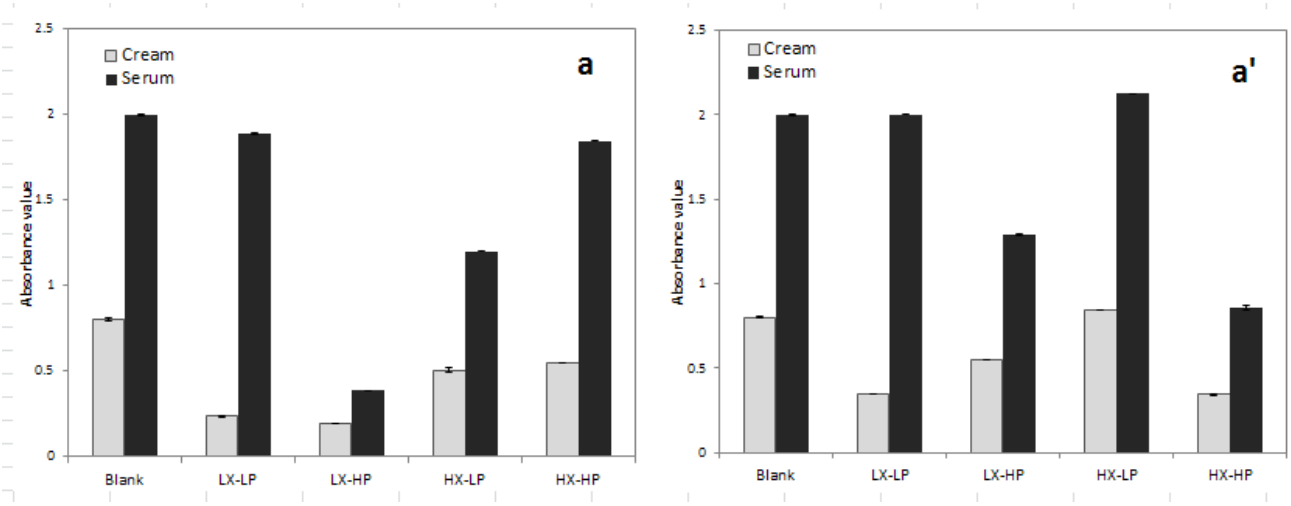

Cream
Serum
a
Absorbance value
2.5
2
1.5
1
0.5
0
Blank
LX-LP
LX-HP
HX-LP
HX-HP
Cream
Serum
a'
Absorbance value
2.5
2
1.5
1
0.5
0
Blank
LX-LP
LX-HP
HX-LP
HX-HP

Figure 7.

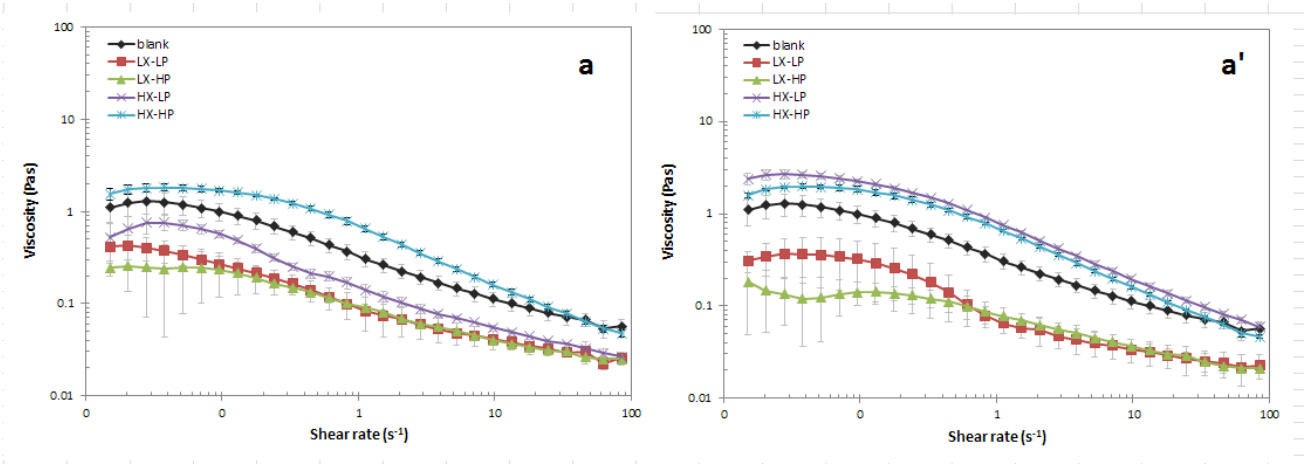

a
a'
blank
LX-LP
LX-HP
HX-LP
HX-HP
Viscosity (Pas)
Shear rate (s-1)
100
10
1
0.1
0.01
0
0
1
10
100

Figure 8.

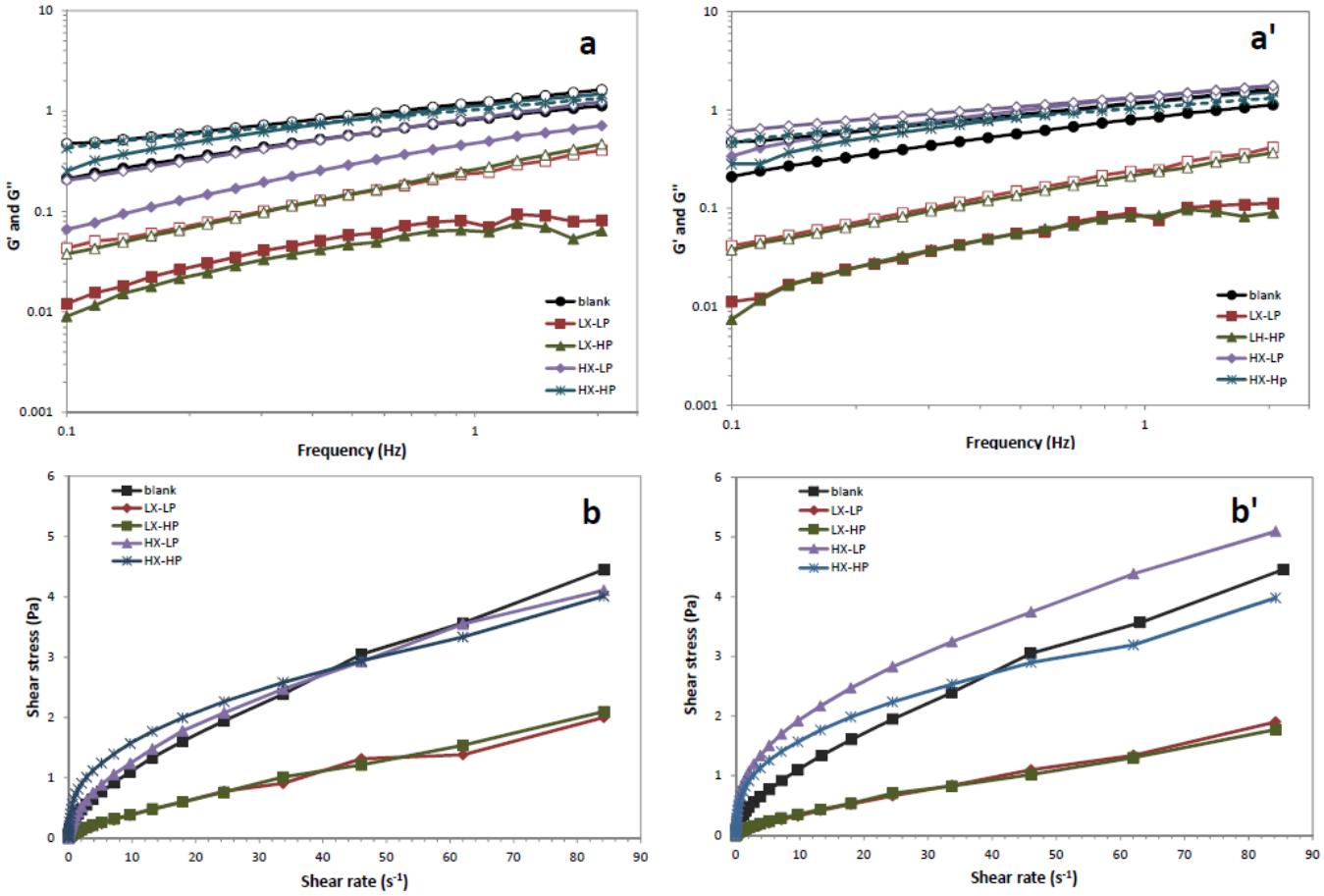
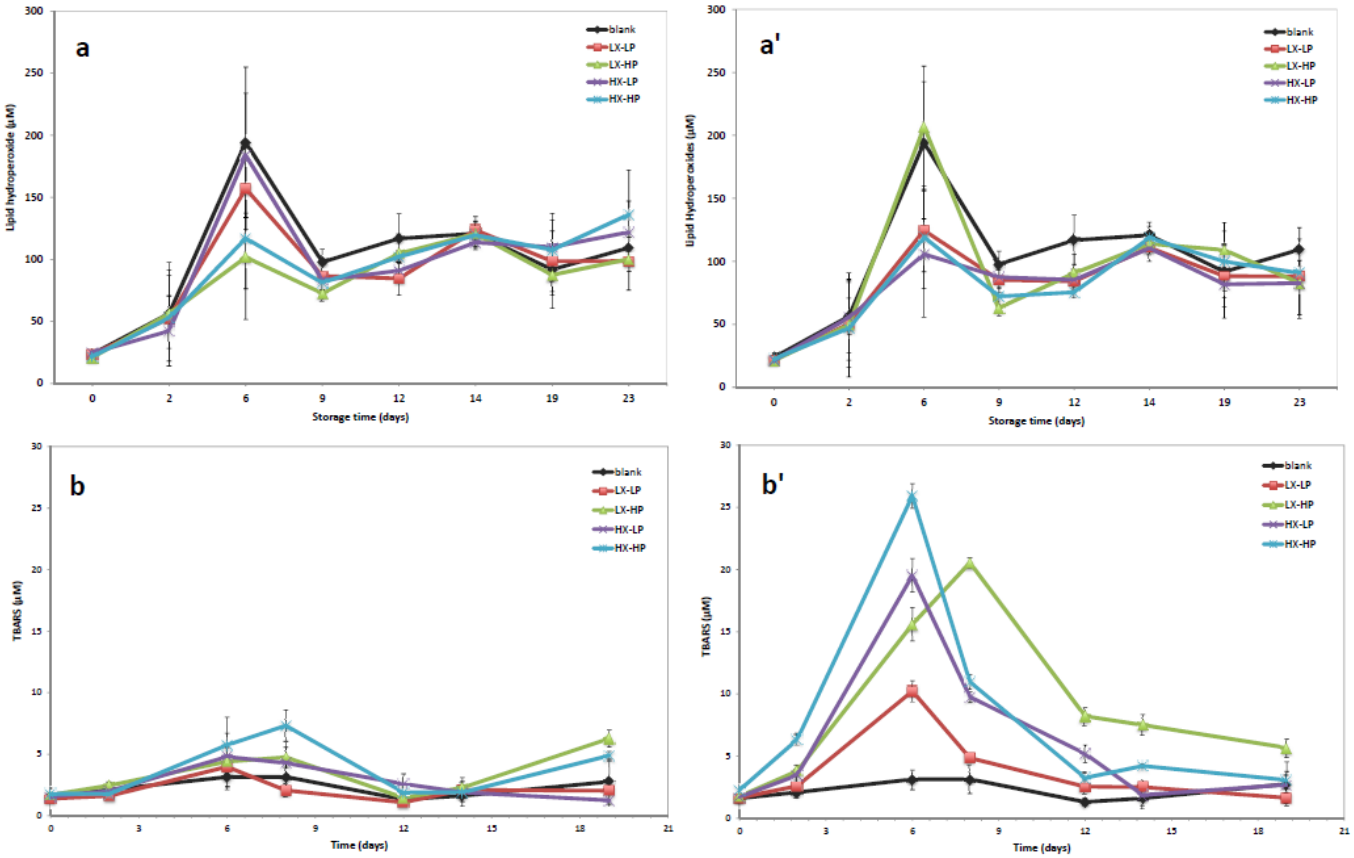

a
G' and G''
Frequency (Hz)
blank
LX-LP
LX-HP
HX-LP
HX-HP
a'
G' and G''
Frequency (Hz)
blank
LX-LP
LH-HP
HX-LP
HX-Hp
b
Shear stress (Pa)
Shear rate (s-1)
blank
LX-LP
LX-HP
HX-LP
HX-HP
b'
Shear stress (Pa)
Shear rate (s-1)
blank
LX-LP
LX-HP
HX-LP
HX-HP

Figure 9.


a
a'
b
b'
blank
LX-LP
LX-HP
HX-LP
HX-HP
Lipid hydroperoxide (µM)
Lipid Hydroperoxides (µM)
TBARS (µM)
Storage time (days)
Time (days)