Olive:linseed:fish oils
(70:20:10 w/w/w)

Double emulsions with an olive leaves extract encapsulated in the internal aqueous phase (DE/OLE)

Meat systems with liquid oil and non-encapsulated OLE (MS-L/OLE)

Meat systems with DE/OLE (MS-DE/OLE)

- Remaining OE content
- △ TBARS
- PV
- * FRAP
- ◇ DPPH

Double emulsions with a purple cactus pear extract encapsulated in the internal aqueous phase

Unstructured: DE-CP

+ Gelatin: DE-CP-G

+ Gelatin + Transglutaminase: DE-CP-GT

Highlights

Olive leaves extract was encapsulated in double emulsion (DE/OLE) for fat replacement

DE/OLE showed good physical stability and high antioxidant capacity during storage

Meat systems with these double emulsions (MS-DE/OLE) showed high oleuropein retention

MS-DE/OLE showed high oxidative stability and antioxidant capacity during storage

The remaining oleuropein content and the antioxidant capacity were highly correlated

Double emulsions with olive leaves extract as fat replacers in meat systems with high oxidative stability

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Abstract

Double emulsions (DE) with a healthy oil blend as lipid phase and an olive leave extract (OLE) encapsulated in the internal aqueous phase (DE/OLE) were incorporated as fat replacers in meat systems, in order to improve both the lipid profile and the oxidative stability. After 14 days of storage at 4 °C, DE/OLE showed good physical stability (90% of globule population was still below 10 µm diameter), and high antioxidant capacity (over 80%), longer than time required for this type of food ingredients. A high correlation was found between the remaining oleuropein content and the antioxidant capacity in both meat systems with DE/OLE (MS-DE/OLE) and meat systems with the oil blend as liquid oil and non-encapsulated OLE (MS-L/OLE). MS-DE/OLE were technologically feasible and showed higher retention of oleuropein (69%), oxidative stability and antioxidant capacity at 60 °C for 7 days than MS-L/OLE, where oleuropein was almost depleted. The encapsulation of OLE in DE could be a suitable strategy to avoid lipid oxidation in meat systems with healthier lipid profile.

Keywords: double emulsions; olive leaves extract; oleuropein; antioxidant activity; meat systems; oxidative stability

1. Introduction

Water-in-oil-in-water ($W_1/O/W_2$) double emulsions (DEs) are multi-compartmentalized systems in which droplets of a water-in-oil (W_1/O) emulsion are dispersed within an outer continuous aqueous phase (W_2) (McClements, Decker, Park, & Weiss, 2009). by suitable formulation of the oil phase, DEs offer the opportunity to design delivery systems for fatty acids that can be used as ingredients in the development of healthier/potentially functional foods, with lower content of saturated fatty acids and higher content of monounsaturated and polyunsaturated fatty acids for the prevention of diet-related chronic diseases (FAO/WHO, 2010; Tressou, Moulin, Vergès, Le Guillou, Simon, & Pasteau, 2016). In recent years, there has been a growing interest in the improvement of the fat content in meat products for meeting consumer demands. In this context, DEs have been proposed as a novel strategy for the improvement of their lipid profile, in replacement of animal fat usually used in this type of food, with high content of saturated fat. Although studies focused on the application of DEs in meat systems are still scarce, the replacement of pork backfat by DEs formulated with olive, perilla or chia oils was technologically feasible in gel/emulsion meat systems (Cofrades, Antoniou, Solas, Herrero, & Jiménez-Colmenero, 2013; Freire, Bou, Cofrades, Solas, & Jimenez-Colmenero, 2016; Cofrades, Santos-López, Freire, Benedí, Sánchez-Múniz, & Jiménez-Colmenero, 2014; Serdaroğlu, Öztürk, & Urgu, 2016; Öztürk, Urgu, & Serdaroğlu, 2017).

Although oil-in-water emulsions can also fulfill this role as delivery system of fatty acids, $W_1/O/W_2$ double emulsions have potential advantages over oil-in-water emulsions such as encapsulation of water soluble bioactive compounds, masking of unpleasant flavors and/or developing of reducing fat-products (McClements et al., 2009; Mohammadi, Jafari, Assadpour, & Esfanjani, 2016a). The encapsulation of antioxidant

compounds in the internal aqueous phase (W_1) of DEs may have two strategic purposes: to protect the oil phase of DE against lipid oxidation, especially in the case of DEs designed with high content of polyunsaturated fatty acids; and, from a nutritional point of view, to optimize the presence of bioactive compounds in foods in which DEs will be incorporated as ingredient.

Furthermore, Olive (*Olea europaea* L.) leaves are actually one of the main by-products generated from the olive oil industry, and they have been used for centuries in folk medicine, generally as infusion with therapeutic properties (Şahin and Bilgin, 2018). Oleuropein, a glucoside ester of elenolic acid and hydroxytyrosol, is one of the major phenolic compounds occurring in olive leaves. Significant health promoting effects have been reported for oleuropein, such as antioxidant, cholesterol lowering, cardioprotective, anti-inflammatory, hypoglycemic or antimicrobial (Şahin and Bilgin, 2018). The wide profile of polyphenols in olive leaves makes the OLE to show a great potential as natural antioxidant. OLE has been incorporated as antioxidant in several food matrices, such as edible oils, frying oils, table olives, meat and meat products (Nunes, Pimentel, Costa, Alves, & Oliveira, 2016; Hayes, Stepanyan, Allen, O'Grady, & Kerry, 2010; 2011). However, when OLE-polyphenols are incorporated in food matrices, they are exposed to adverse conditions in the food (enzymes, pH, interaction with other components) as well as during food processing, which may lead to their degradation (Urzúa, González, Dueik, Bouchon, Giménez, & Robert, 2017). Thus, the encapsulation of OLE-polyphenols may be useful to preserve their activity and also to control their release (time and/or place), as was reported in starchy food matrices (Urzúa et al., 2017; Pacheco, González, Robert, & Parada, 2018). To the best of our knowledge, there are few studies focused on the encapsulation of OLE, especially in DE (Mohammadi et al., 2016a; Mohammadi, Jafari, Esfanjani, & Akhavan, 2016b). Other

methods have been reported for OLE encapsulation, namely spray-drying (Kiritsakis, Goula, Adamopoulos, & Gerasopoulos, 2017), but also freeze-drying (Ganje, Jafari, Dusti, Dehnad, Amanjani, & Ghanbari, 2016) or electrostatic extrusion (Belščak et al., 2011).

The aim of this study was the incorporation of a DE, formulated with a healthy oil blend as lipid phase and an OLE encapsulated in the internal aqueous phase, in meat matrices as fat ingredient in replacement of pork backfat. The stability of DE/OLE as ingredient was evaluated for 14 days at 4 °C. Furthermore, the oxidative stability and the remaining oleuropein content were studied in the meat model systems with DE/OLE; together with their resultant lipid profile and technological feasibility (water and fat binding properties and texture).

2. Materials and methods

2.1. Materials

Olive (*Olea europaea* L.) leaves cv. Arbequina were collected at Melipilla (Chile, 2015). Double emulsions: olive oil (Team Foods S.A., Chile), linseed oil (Nutra Andes Ltda., Chile) and fish oil (Spess S.A., Chile), polyglycerol ester of polyricinoleic acid (PGPR; Dimerco S.A., Chile), sodium caseinate (Prinal S.A., Chile). Pork meat (fresh post-rigor raw pork, mixture of *biceps femoris*, *semimembranosus*, *semitendinosus*, *gracilis* and *adductor*) and pork backfat were purchased in a local market. The reagents 1,1-diphenyl 2-picrylhydrazyl (DPPH) free radical, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ) and oleuropein standard ($\geq 98\%$) were purchased from Sigma–Aldrich (Chile). Fatty acid methyl esters (FAME) standard (GLC 569) was purchased from Nu-Check Prep. Inc. (USA).

2.2. Preparation of OLE

The olive leaves were blanched (95 °C for 4.5 min), dried (45 °C for 18 h) in a forced-air oven (UFE-800, Memmert, Germany) and ground in a knife mill (Wiley Mill, Model-2, A.H. Thomas Co.). The olive leaves extract was prepared according to previous studies (Urzúa et al., 2017), and stored at -20 °C in dark bottles. The total polyphenol content and the oleuropein content of OLE was 47.1 ± 0.4 mg gallic acid equivalent/mL and 39.8 ± 1.0 mg/mL, respectively.

2.3. Preparation of DE

Double emulsions were formulated following a two-step emulsification process (Silva et al., 2018) with slight modifications. OLE was incorporated in the internal aqueous phase (W_1) (357 mg oleuropein/kg DE). A blend of olive, linseed and fish oils (70:20:10 w/w/w) was used as lipid phase (Silva et al., 2018). The osmolarity of W_1 and the external aqueous phase (W_2) was equilibrated with NaCl using an osmometer (3320, Advanced Instruments, USA) to prevent diffusion phenomena. PGPR was added to the oil blend (6% w/w), and a W_1/O coarse emulsion was prepared by drop-wise addition of W_1 (20%) to the lipid phase (80%) using a blender (Thermomix, Vorwerk, Germany; 3250 rpm, 5 min, 50 °C). This W_1/O emulsion was homogenized twice at 55,000 kPa (first stage)/7,000 kPa (second stage) with a two-stage high pressure homogenizer (Panda Plus 2000, GEA Niro Soavi, Italy). The $W_1/O/W_2$ coarse emulsion was prepared by gradually addition of W_1/O (40%) to W_2 (60%) containing sodium caseinate (0.5% w/w) at 700 rpm and room temperature. This $W_1/O/W_2$ coarse emulsion was homogenized twice at 15,000 kPa (first stage)/3,000 kPa (second stage).

2.4. Characterization and stability of DE

2.4.1. Determination of droplet size and size distribution

The size and distribution of oil droplets in DEs were determined using a Malvern Mastersizer 3000 particle size analyzer (Malvern Instrument Ltd., Worcestershire, UK), equipped with a He-Ne laser ($\lambda = 633\text{nm}$) and a measurement range 0.02–2000 μm . Particle size calculations were based on the Mie Scattering theory. Ten measurements were performed on each emulsion. The results were expressed as the average of the volume-weighted mean globule size $D_{4,3}$.

2.4.2. Confocal laser scanning microscopy

Morphology of DEs was evaluated by confocal laser scanning microscopy (CLSM, LSM 700, Carl Zeiss, Germany) at 40x and 100x magnifications. The oil phase of DEs was fluorescently labelled with Nile red (0.02% w/w). Samples were placed onto microscope slides, covered with a cover glass and subjected to 488 nm excitation wavelength, while fluorescent signal was collected at 580 nm. The software used for the CLSM imaging was ZEN 2012 (Blue Edition, Carl Zeiss, Germany).

2.4.3. Encapsulation efficiency

The encapsulation efficiency (EE) of OLE in DEs was estimated by measuring the EE of oleuropein, the major polyphenol in the extract according to previous studies (Urzúa et al., 2017). The EE of oleuropein was determined by measuring the oleuropein concentration in W_2 by HPLC according to Talhaoui, Gómez-Caravaca, León, De la Rosa, Segura-Carretero and Fernández-Gutiérrez (2014), using a Merck Hitachi L-6200 pump, a Waters 996 photodiode-array detector and a C18 column (2.7 μm , 4.6 i.d. x 100 mm, Poroshell 120, Agilent Technologies, USA). Oleuropein was quantified using an oleuropein standard calibration curve (2.5–330 $\mu\text{g/mL}$; $R^2 = 0.99$). A 4-fold dilution of DEs in water with the adjusted osmolarity was centrifuged (2500 g, 30 min) to obtain W_2 , filtered (0.22 μm , Millipore filter), and injected into the HPLC. The EE was defined

as the percentage of oleuropein in W_1 that remained in the primary emulsion W_1/O after the second emulsification process and calculated following the equation (Eq. 1):

$$EE = 100 - (\text{Oleuropein}_{W_2} \times 100) / \text{Oleuropein}_i \quad (1)$$

Where Oleuropein_{W_2} is oleuropein concentration recovered in W_2 , Oleuropein_i is the oleuropein total concentration added to W_1 .

2.4.4. Emulsion stability

Emulsion stability of DEs was evaluated using a vertical scan analyzer (Turbiscan MA2000, Formulation, France), with a pulsed near-IR light source ($\lambda = 850$ nm) and two synchronous detectors (transmission and backscattering). The physical phenomena of destabilization were evaluated through the backscattering (BS) profiles as a function of the sample height in the test cells (60 mm) for 14 days at 4 °C. Creaming index (CI) was calculated from the BS profiles according to the following equation (Eq. 2):

$$\text{Creaming Index} = \frac{HS}{HE} \times 100 \quad (2)$$

Where HS is the height of the serum layer and HE is the total height of the emulsion.

2.4.5. Antioxidant capacity of DE with OLE

The antioxidant capacity of the DE/OLE was determined by DPPH (Brand-Williams, Cuvelier, & Berset, 1995) and FRAP (Benzie and Strain, 1996) methods, both expressed as trolox equivalent (TE)/g DE.

2.5. Preparation of meat systems

Five meat systems (MS) were designed with the same content of fat (10.4 g fat/100 g) and muscle protein (15 g/100 g). The formulation of the different MS is shown as supplementary material. Pork backfat was used as fat ingredient in one of the meat systems (control, MS-C). The improvement of the lipid profile was performed by total

replacement of pork backfat by the same proportion of oil blend (olive:linseed:fish oil 70:20:10 w/w/w), incorporated as either liquid oil (MS-L) or as the lipid phase of DE (MS-DE). OLE was added directly to the meat matrix in meat systems with the oil blend incorporated as liquid oil (MS-L/OLE), and encapsulated in the W_1 of DE in meat systems with the oil blend incorporated as lipid phase of DE (MS-DE/OLE). In both cases, OLE was incorporated to achieve a final oleuropein concentration of 100 mg/kg of meat system that was set according to previous studies (data non-shown). Furthermore, all the meat systems contained sodium chloride (2 g/100 g) and sodium caseinate (0.5 g/100 g). The meat batters were prepared according to Cofrades et al. (2013), using a chilled cutter (Stephan Universal Machine UM5, Stephan u. Söhne GmbH and Co., Germany). The final meat batter temperature was below 12 °C in all cases. The meat batters were placed in tubes (50 mL) that were hermetically closed, centrifuged (2500 g, 4 °C, 5 min) to remove air bubbles and heated (70 °C for 30 min) in a water bath. The tubes were cooled at room temperature and stored at 4 °C for the determination of the fatty acid profile and texture profile analysis (TPA) or at 60 °C for the evaluation of their oxidative stability, antioxidant capacity and remaining oleuropein content.

2.6. Characterization of the meat systems

2.6.1. Fatty acid profile

Lipids were extracted from the meat systems according to Bligh and Dyer (1959), adjusting moisture to 80%, and FAMES were prepared according to UNE-EN ISO 5509:2001. FAMES were analyzed according to Silva et al. (2018), using a gas chromatograph (7890B, Agilent, Chile), with a fused-silica capillary column (HP-88, 100 m x 0.25 mm i.d. x 0.20 µm film thickness) and a flame ionization detector. FAMES were identified by their retention time using a FAME standard, and the results

were expressed as percentage of methyl esters. The determination was performed in triplicate.

2.6.2. Water and fat binding properties

Water and fat binding properties were determined according to Jiménez-Colmenero, Carballo and Solas (1995). The tubes containing the meat batters (8 tubes for each formulation) were opened immediately after heating, and left to stand upside-down for 50 min to recover the released fat and water in a previously weighed plate. Total loss (TL) was determined as the weight loss and expressed as percentage of the initial sample weight. Water loss (WL) was determined as the weight loss after drying the released exudate at 105 °C for 24 h, and expressed as percentage of the total loss. Fat loss (FL) was determined as the difference between TL and WL.

2.6.3. Texture profile analysis

TPA was performed according to Bourne (1978), using a texturometer (BDO-FDO.5T5, Zwick/Roell, Germany). Ten pieces (2.0 cm height and 2.5 cm diameter) from each formulation were axially compressed to 40% of their original height. A 5 kg load cell was used at a crosshead speed of 1 mm s⁻¹ to obtain force-time deformation curves. Hardness was the peak force (N) required for the first compression; cohesiveness was the ratio between active work done under the second compression and that done under the first one (dimensionless); springiness was the distance (mm) the sample recovered after the first compression; chewiness was the product of hardness, cohesiveness and springiness (N x mm). All the measurements were performed at room temperature.

2.6.4. Oxidative stability under accelerated conditions

The meat systems were subjected to an accelerated oxidation study at 60 ± 1 °C in a forced air oven (BE500, Memmert, Germany). Samples (in triplicate) were removed at

specific time intervals to determine the formation of peroxides and thiobarbituric acid reactive substances (TBARS), after a lipid extraction performed according to Bligh and Dyer (1959). Peroxide values were determined according to AOAC (1992), and the results were expressed as mEq O₂/kg of fat. The TBARS were determined as described by Poyato et al. (2015), and the results were expressed as mg malonaldehyde (MDA)/kg of sample.

Furthermore, the remaining oleuropein content and antioxidant capacity of the meat systems, evaluated by DPPH and FRAP, were also periodically determined during the oxidation study. For this, 10 g of each meat system was homogenized (T18, IKA, Germany) with ethanol:water (30 mL; 50:50 v/v) for 1 min, and stirred in an orbital shaker (220 rpm for 1 h). Afterwards, the samples were centrifuged (8500 rpm, 20 min, 10 °C), the supernatant was withdrawn and the extraction was repeated. The supernatants were joined and concentrated in a rotatory evaporator (R100, Büchi, Switzerland) until a final volume of 5 mL. The remaining oleuropein content was determined on this concentrated extract according to Talhaoui et al. (2014) as described in 2.4.2. section, and the results were expressed as mg oleuropein/kg of meat system. The data were fitted by a first-order kinetic model, $C = C_0 e^{-kt}$. Degradation rate constants (k) were obtained from the slope of a plot of the natural log of the remaining oleuropein content vs. time.

Furthermore, the antioxidant capacity of the meat systems was determined by DPPH (Brand-Williams et al., 1995) and FRAP (Benzie and Strain, 1996) on the concentrated extracts, and the results were expressed as µmol TE/g of meat system.

2.7. Statistical analysis

One-way variance analyses were carried out to establish significant differences between mean values obtained for the same sample. The differences of means between samples were resolved by confidence intervals using Fisher Least Significant Difference (LSD) test (Statgraphics 5.0 program, Manugistics Inc., Rockville, MA). The level of significance was set for $p \leq 0.05$.

3. Results and discussion

3.1. Characterization and stability of DE with OLE

3.1.1. Droplet size and droplet size distribution

DE/OLE showed the typical compartmentalized structure of double emulsions, with small water droplets inside the oil globules (supplementary material). Figure 1a shows the droplet size distribution of DE/OLE during 14 days of storage at 4 °C. Initially, DE/OLE showed a dominant population ranging from 0.3 to 20 μm and a small population (1.63%) between 20 and 310 μm . Although an increase in size was observed during storage, possibly due to coalescence of the oil globules, 90% of total globule population was below 10 μm at day 14, indicating good stability. This increase in size led to a significant increase ($p \leq 0.05$) in $D_{4,3}$ values (Table 1) from $3.76 \pm 0.42 \mu\text{m}$ (day 0) to $9.11 \pm 0.57 \mu\text{m}$ (day 14), since this parameter is sensitive to the presence of large particles within a polydisperse system (McClements, 2016). Several factors may affect both droplet size and droplet size distribution in DEs, including type and concentration of emulsifiers, emulsification method, type of oil phase, viscosity of the phases and presence of bioactive compounds (Lamba, Sathish, & Sabikhi, 2015; McClements, 2016; Cofrades et al., 2014). In our previous study, DEs prepared under the same operational conditions and formulation but without OLE in its structure (Silva et al., 2018) showed monomodal droplet size distribution, with lower $D_{4,3}$ values (1.49-2.61

µm during 28 days of storage at 4 °C). Therefore, the encapsulation of OLE in W₁ of DE leads to a slight increase of oil globule sizes, together with a higher increase in size during storage. Souliem, Kobayashi, Neves, Sayadi, Ichikawa and Nakajima (2014) found higher droplet diameters with increasing concentrations of oleuropein (0.1-0.7%) in water-in-oil emulsions. However, the oleuropein concentration did not affect the droplet size distribution of DE prepared from these primary W/O emulsions.

3.1.2. Emulsion stability

The BS profile and CI values of DE/OLE are shown in Figure 1b and Table 1, respectively. A clarification process was observed at the bottom of the cells with storage time (Figure 1b), due to the lower density of oil droplets that rise towards the top of the cell (Matos, Gutiérrez, Iglesias, Coca, & Pazos, 2015), leading to the increase ($p \leq 0.05$) of CI values (Table 1) from $3.20 \pm 0.05\%$ (day 0) to $13.07 \pm 0.88\%$ (day 14). Furthermore, the levels of BS in the middle of the sample were fairly constant during the 14 days of storage, suggesting limited coalescence of the oil droplets. When DE/OLE was compared with a control DE without OLE (Silva et al., 2018), similar CI values were found at day 0 (3.20% and 3.86% for DE/OLE and control DE, respectively), but the encapsulation of OLE in W₁ gave a higher increase in CI values with storage time, in agreement with the results reported for droplet size and droplet size distribution.

3.1.3. Encapsulation efficiency

Initially, the EE of oleuropein was $92.0 \pm 3.1\%$ (Table 1). Although the EE of oleuropein decreased ($p \leq 0.05$) during storage at 4 °C, high values were still found at day 14 ($82.3 \pm 2.6\%$). There are few studies dealing with the encapsulation of polyphenols in DE (Cofrades et al., 2014; Flaizt et al., 2016; Aditya, Aditya, Yang, Kim, Park, & Ko, 2015; Matos, Gutiérrez, Martínez-Rey, Iglesias, & Pazos, 2018; Matos et al., 2015;

Silva et al., 2018), particularly of oleuropein or oleuropein-rich extracts (Souliem et al., 2014; Mohammadi et al., 2016a; Mohammadi et al., 2016b). The EE of bioactive compounds in DEs may depend on the type of bioactive compound, but also on the formulation and technology used to prepare the double emulsion (Souliem et al., 2014; Matos et al., 2018). High values of EE for oleuropein (86.9%) have also been reported by Souliem et al. (2014), although DE was prepared by microchannel emulsification, and the entrapment efficiency of oleuropein was estimated using calcein, with a similar molecular weight. In other study, Mohammadi et al. (2016a) reported the EE of an OLE in a double emulsion as percentage of encapsulated total phenolic compounds, with values ranging from 93.3-96.6% at day 0 to 72.7-88.8% at day 22 of storage at 30 °C.

3.1.4. Antioxidant capacity

Table 1 shows the antioxidant capacity of DE/OLE measured by both FRAP and DPPH during 14 days of storage at 4 °C. DPPH values were affected by the storage time, and a slight but significant decrease ($p \leq 0.05$) from 44.70 ± 0.63 to 37.72 ± 0.84 $\mu\text{mol TE/g}$ DE was found during the 14 days of storage. A similar behaviour was found in FRAP values, with a slight decrease ($p \leq 0.05$) from 2.74 ± 0.02 to 2.38 ± 0.01 $\mu\text{mol TE/g DE}$ during the storage time. This slight loss of antioxidant capacity may be related to the release of bioactive compounds of OLE from W_1 and their role as antioxidants to prevent the lipid oxidation in DE during storage. Therefore, according to the DPPH and FRAP values, DE/OLE maintained a high antioxidant capacity (over 80%) during 14 days of storage, longer time than that required for this type of food ingredients. Although oleuropein and oleuropein-rich extracts have been encapsulated in DE in a few studies (Souliem et al., 2014; Mohammadi et al., 2016a; Mohammadi et al., 2016b), the antioxidant capacity of the resultant DE have not been reported. However, hydroxytyrosol (phenolic compound in olive leaves and olive oil) has been encapsulated

in the inner aqueous phase of DE with chia oil as lipid phase, showing higher DPPH free radical scavenging capacity, ferric reducing ability and oxidative stability than control DE (Cofrades et al., 2014).

3.2. Meat systems

3.2.1. Fatty acid profile

Table 2 shows the fatty acid profile of the meat systems. The oil blend used in this study was low in SFA (15%) and rich in MUFA (59%) and PUFA (26%), with 15% ω 3 and 10% ω 6 (Silva et al., 2018). As expected, the replacement of pork backfat by the oil blend, both as liquid and as oil phase in DE, led to large differences in the fatty acid profile. The content of MUFA was significantly ($p \leq 0.05$) higher in those systems with the oil blend (around 58%) than in the control system (around 42%), due to the high percentage of olive oil (70% of the oil blend). In contrast, MS-C showed the highest content ($p \leq 0.05$) of SFA (37%), especially for stearic (C18:0) and palmitic (C16:0) acids. The content of PUFA was significantly ($p \leq 0.05$) higher in the meat systems with the oil blend (18.86-19.41%) than in the control system (15.63%), where was mainly comprised of linoleic acid (14.93%). In contrast with the control system, all the meat systems with the oil blend showed high contents of ω 3 PUFA (around 10%), with approximately 2% of long chain ω 3 PUFA (eicosapentaenoic acid C20:5 n-3+ docosahexaenoic acid C22:6 n-3), together with more balanced ω 6/ ω 3 ratios. Several studies have reported that the replacement of pork backfat by DE formulated with vegetable oils provides more healthy fatty acid profiles in meat systems (Cofrades et al., 2013; Serdaroğlu et al., 2016; Freire et al., 2016).

3.2.2. Oxidative stability of meat systems, remaining oleuropein content and antioxidant capacity

Figure 2a shows the peroxide values in meat systems under accelerated oxidative conditions. At the beginning of the storage, no significant differences ($p>0.05$) were found in peroxide values among meat systems without OLE (MS-C, MS-DE and MS-L), indicating that the type of fat and mode of incorporation (pork bakfact, double emulsion, liquid oil) did not have a significant effect on peroxide value. However, a large increase in peroxide value was found in MS-C during the storage, reaching values of 25 mEqO₂/kg oil at day 1. This increase may be attributed to the relatively high content of linoleic acid in this sample (14.93%), together with the absence of those antioxidants naturally present in the vegetable oils that contain MS-DE and MS-L. A higher oxidative stability was found in MS-DE and MS-L, although MS-DE reached peroxide values significantly lower ($p\leq 0.05$) than MS-L at the end of the storage period (day 7), suggesting that the structure of multiple emulsion could provide a protective effect on the lipid oxidation since the external aqueous phase may act as a continuous phase covering oil globules and forming a protective barrier (Serdaroğlu et al., 2016). Furthermore, meat systems with non-encapsulated OLE (MS-L/OLE) and OLE encapsulated in W₁ of DE (MS-DE/OLE) showed high oxidative stability until day 5 of storage, with significantly lower peroxide values than MS-C, MS-DE or MS-L ($p\leq 0.05$). However, a large increase of peroxide values was found in MS-L/OLE at day 7 of storage, suggesting the depletion of antioxidant compounds when non-encapsulated OLE was incorporated to the meat matrix.

Figure 2b shows the TBARs values in meat systems under accelerated conditions. The TBARs assay is the most common assay used to evaluate the oxidative stability of meat systems stored at 4° C in studies dealing with the incorporation of DE as fat replacers (Cofrades et al., 2014; Freire et al., 2016; Serdaroğlu et al., 2016; Öztürk et al., 2017). All the meat systems containing the oil blend showed higher TBARs values than the

meat systems with pork backfat (MS-C) until day 5 of storage, when a significant increase of TBARs values was found in MS-C, coinciding with the decrease of peroxide values found at this time of storage (Figure 2a). Cofrades et al. (2014) and Freire et al. (2016) also reported that meat samples where pork backfat was replaced by chia and perilla oils, respectively, showed higher TBARs values than pork backfat samples. Regarding the meat systems with the oil blend and without OLE (MS-L and MS-DE), the mode of oil incorporation had an effect of TBARs values. Thus, meat systems with the oil blend incorporated as DE showed significantly lower values of TBARs than meat systems containing the liquid oil blend from day 3 of storage, suggesting that the structure of double emulsions may hinder the lipid oxidation (Serdaroğlu et al., 2016). In contrast, the mode of incorporation of perilla oil in meat systems (liquid, single emulsion, DE) did not show an effect on lipid oxidation according to Freire et al. (2016). Cofrades et al. (2014) reported that chia oil was more prone to oxidation when it was incorporated as DE in meat systems than when added in the liquid form, which may be related to a higher susceptibility to oxidation of chia oil during the emulsion preparation, given its higher content of polyunsaturated fatty acids compared with the oil blend used in this study.

The incorporation of OLE into the meat systems, both non-encapsulated (MS-L/OLE) and encapsulated in W_1 of DE (MS-DE/OLE), improved the oxidative stability, and MS-L/OLE and MS-DE/OLE showed lower TBARs values than their respective controls (MS-L and MS-DE, respectively) during all the storage period. Furthermore, a higher oxidative stability was found when OLE was encapsulated in W_1 of DE, as also described for peroxide values. To the best of our knowledge this is the first study where an OLE is incorporated into a meat matrix encapsulated in the W_1 of a DE. Non-encapsulated OLE has been incorporated to other meat systems to delay lipid oxidation.

According to Botsoglou, Govaris, Ambrosiadis, Fletouris and Botsoglou (2014), the incorporation of OLE (200 and 300 mg gallic acid equivalent/kg) reduced the production of both hydroperoxides and MDA in frozen n-3 fatty acids-enriched pork patties. OLE also reduced lipid oxidation in both cooked pork patties (200 mg extract/kg; Hayes et al., 2010) and sausages (200 mg extract/kg; Hayes et al., 2011) in comparison with control samples. Alirezalu, Hesari, Eskandari, Valizadeh, and Sirousazar (2017) also reported that the incorporation of OLE to frankfurter type sausage contributed to improve its shelf-life.

3.2.3. Evolution of the remaining oleuropein content in meat model systems

Figures 3a and 3b show the evolution of the remaining oleuropein content together with peroxide values and TBARS in MS-L/OLE and MS-DE/OLE, respectively; whereas Figures 3c and 3d show the evolution of the remaining oleuropein content and the evolution of the antioxidant capacity measured by FRAP and DPPH both in MS-L/OLE and MS-DE/OLE, respectively. The remaining oleuropein content was significantly lower in MS-L/OLE than in MS-DE/OLE from the beginning of the storage, possibly due to the partial degradation of oleuropein during cooking together with higher interactions with other components of the matrix. The remaining oleuropein content significantly decreased with time at 60 °C in both meat systems, following a *pseudo*-first-order kinetic (Cardoso, Guyot, Marnet, Lopes-da-Silva, Renard, & Coimbra, 2005). Degradation rate constants (k) obtained from the slope of a plot of the natural log of the percentage retention of oleuropein vs. time, were 0.045 and 0.065 days⁻¹ for MS-DE/OLE and MS-L/OLE, respectively. In the case of MS-L/OLE, the remaining oleuropein content gradually decreased by 23.5% until day 5 of storage, and it was almost depleted at day 7. The high degradation of oleuropein in MS-L/OLE was related with both the increase of peroxide values and TBARS (Figure 3a) and the loss of

antioxidant activity (FRAP and DPPH; Figure 3c) found at the end of the storage period. However, in the case of MS-DE/OLE, the remaining content of oleuropein decreased from day 2 of storage and only a loss of around 31% was found throughout the storage time. Accordingly, DPPH and FRAP values gradually decreased throughout the storage, and around 54% and 79% of the initial antioxidant capacity for DPPH and FRAP, respectively, was maintained at the end of the storage (Figure 3d), leading to the high oxidative stability found in this sample throughout the storage period (Figure 3b). A high correlation between the remaining oleuropein content and both DPPH and FRAP was found for MS-L/OLE ($R^2 = 0.820$ and 0.954 , respectively) and MS-DE/OLE ($R^2 = 0.817$ and 0.918 , respectively) (supplementary material).

Therefore, DE with OLE encapsulated in W_1 had a positive effect on the oleuropein retention and on the antioxidant activity measured by both FRAP and DPPH during accelerated storage. In this sense, Mohammadi et al. (2016b) reported that the nano-encapsulation of OLE in a DE allowed a higher antioxidant activity of the polyphenols. Furthermore, the incorporation of this DE with OLE to soybean oil increased its oxidative stability in comparison with non-encapsulated OLE.

3.2.4. Water and fat binding properties and texture profile analysis

The type of fat and its mode of incorporation significantly affected the binding properties of the meat systems (Table 3). Thus, meat systems with DE with or without OLE (MS-DE and MS-DE/OLE) showed significantly lower values for TL than MS-C, whereas TL significantly increased when the liquid oil blend was incorporated into the meat matrix in replacement of pork backfat (MS-L and MS-L/OLE). In the case of the incorporation of pork backfat or liquid oil as fat ingredient, a fraction of meat protein should be used as water-in-oil emulsifier, especially in the case of liquid oil since this is highly dispersed in the meat matrix, forming smaller oil drops with larger surface area

covered by meat proteins (Youssef and Barbut, 2011). As a consequence, lower amounts of meat protein were available to form a stable protein network, leading to lower binding properties. However, when a DE was used as fat replacer, more meat protein would be available to form the protein network, as the oil blend is already emulsified by non-meat proteins, leading to an improvement of the binding properties. The improvement of binding properties in gel-emulsion systems when pork backfat is replaced by DE with vegetable oils has been reported in other studies (Cofrades et al., 2013; Serdaroğlu et al., 2016; Öztürk et al., 2017). As regards water and fat losses, all the systems showed similar values, around 92% and 7% of TL, respectively.

Regarding textural properties of meat systems (Table 3), the replacement of pork backfat by DE (with or without OLE) increased slightly, but significantly, the hardness of meat systems. This may be attributed to a better distribution of DE in the meat matrix compared to pork backfat, together with a higher meat protein availability leading to harder gel structures, in line with binding properties (Delgado-Pando, Cofrades, Ruiz-Capillas, Solas, & Jiménez-Colmenero, 2010). Furthermore, meat systems with liquid oil as fat ingredient (MS-L and MS-L/OLE) showed hardness values noticeably higher than MS-C. Although initially the composition of all the meat systems was similar in terms of muscle protein and fat contents, the high TL found in these systems after heating may lead to an increase of protein concentration and more compact protein matrices, explaining the high values found for hardness. A similar trend was found for cohesiveness, springiness and chewiness, where the control system showed the lowest values, followed by meat systems with DE and those with liquid oil as fat ingredient. Different results have been reported regarding the effect of animal fat replacement by DE on the texture of gel-emulsion meat systems. According to Cofrades et al. (2013), hardness values increased when pork backfat was replaced by DE with olive oil.

However, Freire et al. (2016) did not found differences in hardness between frankfurters with pork backfat and those with perilla oil, added as DE or liquid oil; although these last two meat systems showed higher values for cohesiveness and springiness. Besides the type of lipid source and the incorporation strategy, other factors such as type of hydrophilic emulsifier in DE, moisture and protein content, and amount of protein available to form stable gels have been reported to affect the textural properties of meat systems (Cofrades et al., 2013; Freire et al., 2016; Serdaroğlu et al., 2016; Öztürk et al., 2017). PGPR has been used as lipophilic emulsifier in all these studies focused on the replacement of animal fat by DE in meat systems (Cofrades et al., 2013; Freire et al., 2016; Serdaroğlu et al., 2016; Öztürk et al., 2017), since this compound is one of the most common and effective emulsifiers used in the development of DE (Muschiolik & Dickinson, 2017). However, currently PGPR is not permitted in meat based products according the European regulation (Commission Regulation No 1129/2011).

4. Conclusions

Double emulsions with OLE encapsulated in W_1 showed good physical stability and high antioxidant capacity during storage at 4 °C for 14 days, longer than time required for this type of food ingredients. The incorporation of OLE in a meat matrix both non-encapsulated and encapsulated in W_1 of DE improved the oxidative stability of the meat systems during accelerated storage, but in a higher extent in the case of encapsulated OLE. The encapsulation of OLE in DE hindered the oleuropein degradation, leading to meat systems with lower contents of both peroxide values and TBARS, as well as higher antioxidant capacity with respect to meat systems with non-encapsulated OLE. In this study, the replacement of pork backfat by olive:linseed:fish oil DE with OLE led to stable meat matrices with healthier fatty acid profile, with noticeable reductions in the SFA content and higher proportions of $\omega 3$ PUFA.

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Figure Captions

Figure 1. Size distribution (1a) and backscattering profile for DE/OLE (1b) during storage at 4 °C for 14 days. Day 0 (—), day 7 (-----), day 14 (—·—).

Figure 2. Peroxide values (2a) and TBARS values (2b) for meat systems with pork backfat (MS-C, ●), oil blend as liquid oil (MS-L, ■), oil blend as lipid phase of DE (MS-DE, ○), oil blend as liquid oil and non-encapsulated OLE (MS-L/OLE, □) and oil blend as lipid phase of DE with OLE encapsulated in the internal aqueous phase (MS-DE/OLE, ▲) during storage at 60 °C for 7 days.

Figure 3. Evolution of the remaining oleuropein content (●) together with peroxide values (□) and TBARS (Δ) in MS-L/OLE (3a) and MS-DE/OLE (3b). Evolution of the remaining oleuropein content (●) and antioxidant capacity measured by FRAP (◇) and DPPH (▽) both in MS-L/OLE (3c) and MS-DE/OLE (3d).

Figure 1

Figure 2

Figure 3

Table 1. $D_{4,3}$ values, Creaming Index (CI), encapsulation efficiency (EE), FRAP and DPPH for DE/OLE during storage at 4 °C.

Time (days)	$D_{4,3}$ (μm)	CI (%)	EE (%)	FRAP (μmol TE/g DE)	DPPH (μmol TE/g DE)
0	3.65±0.25 ^a	3.20±0.05 ^a	92.0±3.1 ^a	2.74±0.02 ^a	44.70±0.63 ^a
7	3.37±0.20 ^a	8.07±0.38 ^b	86.4±2.3 ^{ab}	2.43±0.01 ^b	41.55±0.93 ^b
14	9.11±0.57 ^b	13.07±0.88 ^c	82.3±2.6 ^b	2.38±0.01 ^c	37.72±0.84 ^c

Different letters (a-c) in each column mean significant differences among sampling days ($p \leq 0.05$).

Table 2. Fatty acid composition of the meat systems

Fatty acid	Meat systems				
	MS-C (%)	MS-L (%)	MS-DE (%)	MS-L/OLE (%)	MS-DE/OLE (%)
C14:0	1.29 ± 0.01 ^c	0.95 ± 0.03 ^b	0.89 ± 0.04 ^{ab}	0.83 ± 0.06 ^a	0.82 ± 0.06 ^a
C16:0	22.98 ± 0.08 ^c	14.87 ± 0.22 ^{ab}	15.05 ± 0.09 ^b	14.65 ± 0.06 ^a	14.63 ± 0.15 ^a
C18:0	12.89 ± 0.08 ^b	4.95 ± 0.07 ^a	5.04 ± 0.06 ^a	4.97 ± 0.27 ^a	5.19 ± 0.31 ^a
C16:1	1.99 ± 0.01 ^b	1.95 ± 0.10 ^b	1.85 ± 0.08 ^{ab}	1.81 ± 0.09 ^{ab}	1.70 ± 0.07 ^a
C18:1 n-9	37.70 ± 0.12 ^b	53.12 ± 0.35 ^a	53.82 ± 0.48 ^a	53.76 ± 0.63 ^a	53.53 ± 0.59 ^a
C18:1 n-7	2.52 ± 0.01 ^a	2.70 ± 0.09 ^{bc}	2.78 ± 0.03 ^c	2.75 ± 0.05 ^c	2.59 ± 0.06 ^{ab}
C18:2 n-6	14.93 ± 0.03 ^b	8.56 ± 0.19 ^a	8.59 ± 0.08 ^a	8.37 ± 0.22 ^a	8.66 ± 0.14 ^a
C18:3 n-3	0.70 ± 0.01 ^a	8.88 ± 0.12 ^b	8.88 ± 0.11 ^b	8.65 ± 0.55 ^b	8.58 ± 0.57 ^b
C20:5 n-3	-	1.19 ± 0.07 ^a	1.15 ± 0.06 ^a	1.09 ± 0.10 ^a	1.12 ± 0.08 ^a
C22:6 n-3	-	0.78 ± 0.04 ^a	0.73 ± 0.02 ^a	0.75 ± 0.04 ^a	0.73 ± 0.03 ^a
Σ SFA	37.16 ± 0.15 ^b	20.77 ± 0.26 ^a	20.97 ± 0.01 ^a	20.45 ± 0.28 ^a	20.64 ± 0.40 ^a
Σ MUFA	42.21 ± 0.11 ^a	57.77 ± 0.54 ^b	58.45 ± 0.42 ^b	58.32 ± 0.61 ^b	57.82 ± 0.58 ^b
Σ PUFA	15.63 ± 0.03 ^a	19.41 ± 0.18 ^b	19.35 ± 0.27 ^b	18.86 ± 0.90 ^b	19.09 ± 0.82 ^b
Σ n-3	0.70 ± 0.00 ^a	10.85 ± 0.01 ^b	10.76 ± 0.19 ^b	10.49 ± 0.68 ^b	10.43 ± 0.68 ^b
Σ n-6	14.93 ± 0.03 ^b	8.56 ± 0.19 ^a	8.59 ± 0.08 ^a	8.37 ± 0.22 ^a	8.66 ± 0.14 ^a
PUFA/SFA	0.42 ± 0.00 ^a	0.91 ± 0.01 ^b	0.92 ± 0.01 ^b	0.92 ± 0.06 ^b	0.93 ± 0.06 ^b
n-6/n-3	21.33 ± 0.04 ^b	0.79 ± 0.02 ^a	0.80 ± 0.01 ^a	0.80 ± 0.03 ^a	0.83 ± 0.04 ^a

Different letters (a-c) in each row indicate significant difference among meat systems.

Table 3. Water and fat binding properties and texture profile analysis of meat systems

Meat system	Water and fat binding properties			Texture profile analysis			
	TL (%)	WL (%)	FL (%)	Hardness (N)	Cohesiveness (dimensionless)	Springiness (mm)	Chewiness (N mm)
MS-C	6.06 ± 0.94^b	92.2 ± 0.4^b	7.81 ± 0.36^b	25.77 ± 1.02^a	0.46 ± 0.03^a	4.60 ± 0.14^a	55.75 ± 5.32^a
MS-L	16.02 ± 0.40^d	91.7 ± 0.4^a	8.27 ± 0.42^c	40.46 ± 2.12^d	0.52 ± 0.01^c	5.62 ± 0.07^c	118.14 ± 6.01^d
MS-DE	2.99 ± 0.29^a	92.5 ± 0.4^b	7.54 ± 0.38^b	28.11 ± 2.38^b	0.49 ± 0.02^b	5.25 ± 0.03^b	72.31 ± 8.07^b
MS-DE/OLE	2.44 ± 0.22^a	92.3 ± 0.3^b	7.72 ± 0.40^b	29.33 ± 1.76^b	0.49 ± 0.02^b	5.16 ± 0.08^b	74.15 ± 5.04^b
MS-L/OLE	12.33 ± 0.52^c	93.0 ± 0.2^c	7.00 ± 0.18^a	35.60 ± 1.82^c	0.51 ± 0.01^{bc}	5.59 ± 0.08^c	91.68 ± 4.16^c

Different letters (a-d) in each column mean significant differences among samples ($p \leq 0.05$).

Formulation of the meat systems (g/kg)

Meat system	Meat	Backfat	Oil blend	DE	DE-OLE	OLE	Water
MS-C	695	90	0	0	0	0	190
MS-DE	695	0	0	280	0	0	0
MS-L	695	0	84	0	0	0	196
MS-DE/OLE	695	0	0	0	280	0	0
MS-L/OLE	695	0	84	0	0	2.5	193.5

Micrographs based on confocal laser scanning microscopy of DE/OLE at day 0 at 40x magnification (A) and 100x magnification (B)