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Comparison of simple, double and gelled double emulsions as hydroxytyrosol and n-3 fatty acid delivery systems

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ABSTRACT

The purpose of this study was to compare three different emulsion-based systems, namely simple emulsion, double emulsion and gelled double emulsion, for delivery of n-3 fatty acids (perilla oil at 300 g/kg) and hydroxytyrosol (300 mg/kg). Considering that their structural differences may affect their physical and oxidative stability, this was studied by storing them at 4 °C for 22 days in the dark. The results showed that the oxidative status was maintained in all systems by the addition of hydroxytyrosol. However, there was some loss of hydroxytyrosol, mainly during sample storage and during preparation of the gelled double emulsion. Moreover, the antioxidant loss was more pronounced in more compartmentalized systems, which was attributed to their increased surface area. However, the double emulsion was found to be less stable than the gelled emulsion. Overall, the encapsulation of labile compounds in more complex systems needs to be carefully studied and adapted to specific technological and/or nutritional requirements.

1. Introduction

In recent years, different systems have been proposed for the delivery of hydrophobic and hydrophilic bioactive compounds in foods (Dickinson, 2012; Garti, 1997; Sagalowicz & Leser, 2010). In this context, oil-in-water (O/W) emulsions, hereafter also called simple emulsions (SEs), have been widely used for the delivery of hydrophobic compounds such as tocopherol and n-3 fatty acids in foods and beverages (Lee et al., 2006; McClements, Decker, & Park, 2009; Sagalowicz & Leser, 2010). Multiple or water-in-oil-in-water (W/O/W) double emulsions (DEs) are more complex liquid dispersions in which oil globules containing small water droplets are dispersed in an aqueous continuous phase. This type of emulsion offers a number of promising opportunities for the food industry, e.g. the delivery of hydrophobic compounds in aqueous systems, it allows for the encapsulation of hydrophilic bioactive compounds and can help improve the fat content of foods (by reducing fat and providing a healthier fatty acid profile) (Jiménez-Colmenero, 2013).

In fact the consumption and development of food products enriched with n-3 fatty acids is of great interest in the context of Western diets in view of a variety of reported beneficial health effects, mainly relating to cardiovascular and inflammatory diseases (Ruxton, Reed, Simpson, & Millington, 2004; Simopoulos, 2006). In this regard, the use of perilla oil is of special interest as it is one of the richest terrestrial sources of linolenic acid (Ciftci, Przybylski, & Rudzinska, 2012). However, systems with high n-3 fatty acid contents are prone to oxidation and hence need to be protected, since that oxidation causes loss of nutritional properties and poses a potential hazard in that various of the compounds thus formed have been associated with neurodegenerative and cardiovascular diseases (Esterbauer, Wag, & Puhl, 1993; Perluigi, Coccia, & Butterfield, 2012).

Addition of antioxidants offers a strategy to tackle this problem (Decker, Elias, & McClements, 2010b), especially if these are natural compounds. In this regard, there are some phenolic compounds that are naturally present in olive oils and have been reported to act as antioxidants (Tripoli et al., 2005). These have two advantages. Firstly, they may provide protection to food sensitive components such as n-3 fatty acids during processing and storage. And secondly, they can exert beneficial effects *in vivo* after food consumption. In this connection, hydroxytyrosol (HTy), a major

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phenolic compound in olive oils (averaging 200–500 mg/kg), has recently been receiving attention for the wide range of its biological activities and its capacity to protect against cardiovascular disease (Bulotta et al., 2014; Tripoli et al., 2005). Therefore, the addition of HTy is not only of nutritional benefit when added in sufficient amounts but also of technological utility as it minimizes the oxidation extent (Cofrades et al., 2011; Pazos, Alonso, Sanchez, & Medina, 2008). Recently, Cofrades et al. (2014) studied the oxidative stability of DEs containing chia oil and HTy and that of a cooked meat system prepared with these DEs. These authors reported that DEs showed good antioxidant capacity in the early days of storage; and similarly, DEs containing HTy protected cooked meat systems against oxidation better than DEs without HTy. However, the addition of HTy appeared to exert less antioxidant effect when incorporated in this way than when added to the meat system directly. In fact it was unclear whether these differences were caused by the encapsulation itself or by a reduction in the amount of HTy, which can occur during preparation or storage of DEs as a consequence of the increased surface area. It is therefore important to know the amount of HTy that is encapsulated within the DE and how much is released and lost during preparation and storage.

Novel proposals for liquid phase oil stabilization and structuring (e.g. multiple emulsions, multilayer emulsions, Pickering stabilization) have recently been reviewed (Jiménez-Colmenero et al., 2015; Mao & Miao, 2015). A number of them can be used to improve the quality of reformulated (healthier) foods. For instance, gelled emulsions can be used in different food applications to improve their nutritional, functional and/or sensory characteristics (Li, Cheng, Tatsumi, Saito, & Yin, 2014; Lobato-Calleros et al., 2008; Weiss, Scherze, & Muschiolik, 2005). Gelled DEs (GDEs) thus offer a range of interesting possibilities for the food industry in that they may serve at once to improve the lipid composition, encapsulate bioactive compounds and provide certain plastic properties. Their physical and structural characteristics (stability, viscoelasticity, encapsulation efficiency, phase separation minimization, etc.) offer technological advantages with important effects on the desired quality attributes of foods when used as intermediate products (food ingredients) to optimize the presence of bioactive compounds. Additionally, this strategy may influence the bioavailability and oxidizability of bioactive compounds, linked as these are to their molecular structure and physicochemical properties.

Given that the physical and oxidative stability of emulsion-based systems depends on their structural differences, the aim of this study was to characterize three emulsion-based systems (SEs, DEs and GDEs) for delivery of n-3 fatty acids (perilla oil) and HTy. The oxidative stability was followed over 22 days of storage at 4 °C in the dark. It is crucial to gain an understanding of the behaviour of these systems during storage, as this needs to be considered when designing a delivery system for a particular food application. In this case it will further help to understand the changes that these potential emulsion-based delivery systems may undergo as part of a food product (e.g. meat matrix) and hence estimate how this may affect the reformulated product's characteristics. As far as the authors are aware there have been no studies on the encapsulation process of HTy in PUFA-enriched gelled DEs.

2. Material and methods

2.1. Materials and Reagents

HTy (purity $\geqslant$ 99%) was purchased from Seprox Biotech (Madrid, Spain) and perilla oil (fatty acid composition: 6% 16:0, 2% 18:0, 12% 18:1, 14% 18:2, 65% 18:3) was purchased from Grupo Nutracéutico Chiasa, SL (Meliana, Spain). Upon arrival this oil was stored at 4 °C until the elaboration of the different emulsions systems (less than 1 month) and its hydroperoxide content was 0.22 ± 0.01 mmol cumene hydroperoxide/kg (Shantha & Decker, 1994). The thiobarbituric acid-reactive substances (TBARS) value was under the limit of detection (Cofrades et al., 2014). Sodium caseinate (Excellion EM 7) was purchased from FrieslandCampina DMV (Veghel, The Netherlands), polyglycerol polyricinoleate (PGPR) was purchased from Bavaro Chemicals S.L. (Sant Cebrià de Vallalta, Spain), gelatine (type B, 200–220 bloom) from Manuel Riesgo, S.A. (Madrid, Spain), and microbial transglutaminase (Activa GS) from Ajinomoto (Tokyo, Japan). According to the supplier the activity of the enzyme was 47–82 units of hydroxamate per g. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ), iron(III) chloride hexahydrate, 2,4-dinitrophenylhydrazine (DNPH) and orthophosphoric acid were acquired from Sigma-Aldrich (Madrid, Spain). MilliQ water was used throughout. All other reagents and solvents used were of a suitable grade for spectrophotometric or chromatographic analyses.

2.2. Preparation of simple emulsions (SE), double emulsions (DE) and gelled double emulsions (GDE)

The aqueous phase (W) of the SE consisted of 0.584 g NaCl, 0.04 g sodium azide, 0.441 g sodium caseinate and 44.1 mg HTy dissolved in 100 mL distilled water. The lipid phase (O) consisted of perilla oil (94 g/100 g) plus PGPR (6 g/100 g). The simple O/W coarse emulsion was prepared by gentle addition of the lipid phase (32 g/100 g) to the outer (68 g/100 g) aqueous phase (W) in a Thermomix food processor (TM-31 Vorwerk, Wuppertal, Germany) set at 37 °C on speed 3. Sample was then passed twice through a two-stage high pressure homogenizer (Panda Plus 2000, GEA Niro Soavi, Parma, Italy) at 15 and 3 MPa (first and second stage respectively), and the SE was collected in a beaker immersed in ice. This sample is hereafter designated SE-HTy-PO. Sample aliquots were then transferred to screw-capped tubes and immediately stored at 4 °C in the dark.

A two stage procedure was used to prepare stable DEs, as reported elsewhere (Bou, Cofrades, & Jimenez-Colmenero, 2014b) with minor modifications. The inner (W_1) phase consisted of 0.584 g NaCl plus 375 mg HTy and 0.04 g sodium azide in 100 mL distilled water. The outer (W_2) phase was prepared by dispersing 0.584 g NaCl, 0.04 g sodium azide and 0.5 g sodium caseinate in 100 mL distilled water at room temperature until fully dissolved. The lipid phase (O) used was the same as in the SE. The primary coarse emulsion (W_1/O) was prepared by gentle addition of the inner (W_1) aqueous phase (20 g/100 g) to the lipid phase (80 g/100 g) in the Thermomix food processor set at 37 °C for 15 min on speed 6 (3250 rpm). This primary coarse emulsion was passed twice through a two-stage high pressure homogenizer (Panda Plus 1000) at 55 and 7 MPa respectively and collected in a beaker immersed in ice. The resulting primary fine emulsion (W_1/O) was immediately used for preparation of the DE by gradual addition (40 g/100 g) to the outer (60 g/100 g) aqueous phase (W_2) in the Thermomix food processor set at 37 °C, speed 3 (700 rpm). The resulting coarse W_1/O/W_2 emulsions were passed twice through a two-stage high pressure homogenizer (Panda Plus 1000) at 15 and 3 MPa respectively to obtain the final DE, which was collected in a beaker in ice. This sample is designated DE-HTy-PO. Sample aliquots were then transferred to screw-capped tubes and immediately stored at 4 °C in the dark. The pH of these systems was measured (1/1 v/v, sample/distilled water) using an Orion Research 720A pH meter (Instrumentación Analítica SA, Madrid, Spain). Values were in the range 6.8–7.0.

The GDEs were prepared by mixing the freshly prepared DE with gelatine (4% of the initial weight of the DE). The emulsion

was heated at 40 °C for 15 min while mixing with an overhead stirrer (AGV-8 Bunsen, Madrid, Spain) to dissolve the gelatine completely. This was then cooled down (on ice) to 35–37 °C. At this temperature, transglutaminase (2%) was added gradually while stirring for an extra 2 min after complete homogenization (15–20 min in total). This sample is designated GDE-HTy-PO. Sample aliquots were transferred to 50 mL capacity bottles, screw capped and immediately stored at 4 °C to form the final GDE in the dark. The pH of all gels was measured on a homogenate of sample in distilled water (1/1 v/v). Values ranged between 7.4 and 7.5. In order to examine the antioxidative effect of HTy in this system, a GDE without HTy was prepared and this was compared with another GDE in which the concentration of HTy in its W_1 was of 100 mg/kg.

Note that the technological aptitude should be studied in each particular food application. Accordingly, we mimicked typical refrigeration conditions in the food industry. In this connection, it is worth to mention that the physical appearance of GDE heated for 30 min at 80–90 °C was found to remain unchanged due to the formation of covalent bonds through transglutaminase activity whereas the stability of similar DE against thermal treatments has been studied previously (Bou, Cofrades, & Jimenez-Colmenero, 2014a; Bou et al., 2014b).

2.3. Microscopy

The morphology of the three systems was examined by light microscopy. After 1 and 22 days of storage at 4 °C, SE-HTy-PO and DE-HTy-PO samples were diluted 8–10 times with 5.84 g/L NaCl, placed on microscope slides and carefully covered with a cover slip just before observation. Because of its viscoelastic properties, the GDE-HTy-PO system could not readily be prepared for observation at these storage times. These samples were therefore placed on microscope slides immediately after preparation and before gelation, and carefully covered with a cover slip. They were then stored at 4 °C for 1 and 22 days. The microstructure of these emulsion-based systems was observed using a Leica AF6000 LX (Wetzlar, Germany) at 100X and 160X magnifications.

2.4. Particle size characteristics

The particle size and distribution of oil droplets in SE-HTy-PO and DE-HTy-PO samples was determined immediately after 10-fold dilution with 5.84 g/L NaCl, using a Malvern Mastersizer S particle size analyser (Malvern Instrument Ltd, Worcestershire, UK) equipped with a He-Ne laser (λ = 633 nm). The measurement range was 0.05–900 μm. Obscuration was in the range 8–15%. Particle size calculations were based on the Mie Scattering theory. Volume average diameter (d_{43}) was measured immediately after addition to the dispersion unit. Measurements were carried out in triplicate on each emulsion.

2.5. Gravitational stability

The creaming stability of SE-HTy-PO and DE-HTy-PO at 4 °C was measured in quadruplicate from 20 mL sample aliquots placed in flat-bottomed screw-capped glass tubes. The phase separation (creaming) of SE-HTy-PO and DE-HTy-PO was recorded over storage time at 4 °C in terms of phase separation and expressed as % of total sample height.

2.6. Determination of the HTy content and encapsulation efficiency and stability

In the cases of SE-HTy-PO and DE-HTy-PO, 1 mL of sample (density was checked and this is rounded to 1 g/mL) was mixed with 100 μL of 85% orthophosphoric acid. In the case of GDE-HTy-PO, 5 g of sample was homogenized using an Ultra-Turrax device (Ultra-Turrax T25, IKA-Werke, Staufen Germany) for 30 s at setting 4 (7800 rpm) with 10 ml of 15% trichloroacetic acid dissolved in methanol. Samples were then centrifuged (Consul 21 R Ortoalresa centrifuge, Madrid, Spain) at 2000*g* for 10 min and the supernatant was collected for analysis. HTy was analysed in the clear extract using an Agilent 1200 liquid chromatographic system equipped with an autosampler, quaternary pump and diode-array detector (DAD). A 250 mm × 4.6 mm i.d., 5-μm particle size Nucleosil 120 RP-18 column (Teknokroma) was used, preceded by a ODS precolumn. Elution was performed at a flow rate of 1.0 mL/min, using a mixture of 1% (v/v) formic acid in deionized water (solvent A), acetonitrile (solvent B) and methanol (solvent C) as mobile phase. The solvent gradient changed from 95% A – 2.5% B – 2.5% C to 70% A – 15% B – 15% C in 25 min, to 0% A – 50% B – 50% C in 5 min, to 95% A – 2.5% B – 2.5% C in 5 min, followed by 5 min maintenance. Chromatograms were acquired at 280 nm. Injection volume was 20 μL. For quantification of HTy, a correction factor of 1.06 was applied in the GDE-HTy-PO (gelatin plus transglutaminase addition) and a standard calibration curve was prepared in a range of concentrations from 0.5 to 300 mg/kg to cover all samples. A linear response was obtained throughout the calibration range.

The recovery yield (Ry) was determined to measure the amount of HTy that was recovered in the SE-HTy-PO after preparation. This was calculated from the results in SE-HTy-PO at the initial time as follows:

$$\text{Ry } (\%) = \frac{W}{W_T} \times 100$$

where W is the amount of HTy found in the SE-HTy-PO at the initial time and W_T is the added amount of HTy (300 mg/kg).

The encapsulation efficiency (EE) for HTy in DE-HTy-PO was measured by 4-fold dilution of the DE-HTy-PO after preparation with a 5.84 g/L aqueous solution of NaCl. Samples were then centrifuged at 1300*g* for 15 min (Consul 21 R Ortoalresa centrifuge, Madrid, Spain) to separate the fat globules from the outer aqueous phase. Note that the centrifugation conditions did not affect the emulsion droplet size. EE was defined as the percentage of HTy remaining in the primary emulsion (W_1/O) after the second emulsification step. The amount of HTy entrapped in the DE-HTy-PO and GDE-HTy-PO was considered the compound reservoir and was calculated as follows:

$$\text{EE } (\%) = 100 - \left(\frac{W_2}{W_T} \times 100\right)$$

where W_2 is the amount of HTy found in the outer phase of DE-HTy-PO after preparation (day 0), and W_T is the amount of HTy added (300 mg/kg).

Encapsulation stability (ES) was defined as the release of HTy during storage and was calculated with respect to the amount of HTy remaining in the primary emulsion, using the following equation:

$$\text{ES } (\%) = 100 - \left(\frac{W_{2(t)}}{W_{(t)}} \times 100\right)$$

where $W_{(t)}$ and $W_{2(t)}$ are the total amount of HTy determined in the entire and outer phase of the DE-HTy-PO respectively, at a specific storage time (t).

2.7. Antioxidant capacity

The reducing capacity of samples during storage was determined by the FRAP assay (Pulido, Bravo, & Saura-Calixto, 2000) setup in a plate reader-based automated analysis. The reducing power of samples was measured as the increase in absorbance at

595 nm of the complex tripyridyltriazine/Fe (II) in the presence of samples containing HTy. 10 μL of either Trolox or test sample or water as blank were added to a 96-well microplate followed by the addition of 30 μL of distilled water and 200 μL of 0.3 M acetate buffer, pH 3.6. A microplate reader (Bio-Tek, Winooski, VT, USA) was programmed to record every 90 s for 30 min at 595 nm after the addition of 60 μL of FRAP reagent, which was freshly prepared by mixing 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3$ and 7.5 mL of 0.3 M acetate buffer, pH 3.6. The temperature was maintained at 37 °C. Each value is the average of four determinations. Trolox was used as a standard and results were expressed as μM of Trolox equivalents.

2.8. Lipid oxidation

Malondialdehyde content was determined in its hydrazone by high-performance liquid chromatography (HPLC) using dinitrophenylhydrazine (DNPH) for derivatization (Mateos, Goya, & Bravo, 2004). Sample aliquots (500 μL) were weighed in Eppendorf tubes, to which 100 μL of 6 N NaOH was added. Samples were then sealed and incubated 30 min at 60 °C. After cooling to room temperature, 250 μL of 35% $HClO_4$ was added to the tubes, which were then centrifuged at 12000*g* for 10 min (Eppendorf, Centrifuge 5417R). A 125 μl volume of supernatant was transferred to an Eppendorf vial and mixed with 12.5 μl DNPH prepared as a 5 mM solution in 2 M hydrochloric acid. Finally, this reaction mixture was incubated for 30 min at room temperature protected from light. An aliquot of 50 μL of this reaction mixture was injected onto the HPLC system. HPLC analyses were performed in an Agilent 1100 liquid chromatographic system equipped with a diode array UV–vis detector. A Nucleosil 100 RP-18 column (4.0 × 125 mm) with 5 μm particle size (Agilent) was used, preceded by a Lichrospher precolumn of the same material as the stationary phase (4.0 mm × 4.0 mm). An Agilent Chemstation was used to control all the equipment software systems and perform the data processing. Elution was performed isocratically with a mixture of 0.2% (v/v) acetic acid in deionised water and acetonitrile (62:38, v/v) at a flow rate of 0.6 ml/min at room temperature. Chromatograms were acquired at 310 nm. Alternatively, the TBARS method was also used (Cofrades et al., 2014). In both cases, malondialdehyde was used as a standard and results were expressed as nmol/g or as mg/kg interchangeably.

Lipid hydroperoxides were measured by homogenizing 1 g of sample with 15 ml of chloroform/methanol (1:1, v/v) for 30 s at 4 °C. Subsequently, this solution was mixed with 3.5 ml of 0.5% NaCl and centrifuged at 3000*g* for 30 min at 4 °C. The content in lipid hydroperoxides was measured in the chloroform phase (or directly in perilla oil) as described elsewhere (Shantha & Decker, 1994). Cumene hydroperoxide (CHP) was used as standard and results were expressed as equivalents of mmol CHP/kg.

2.9. Instrumental colour

The colour parameters of SE-HTy-PO, DE-HTy-PO and GDE-HTy-PO were analysed throughout storage. Four sample aliquots of 30 mL were poured into Petri dishes and the surface colour of the bottom was then measured by determining L^*, a^* and b^* using a CIELab scale, where L^* is the parameter that measures lightness, b^* is the tendency towards yellow (yellowness) and a^* the tendency towards red (redness). Measurements were performed on a CR-400 Chroma Meter (Konica Minolta Business Technologies, Inc., Tokyo, Japan). The instrument was set for standard illuminant D-65 and a 2° observer, and standardized using a standard white plate.

2.10. Statistical analysis

All experiments were carried out in triplicate. Series of one-way ANOVAs were performed for each factor (emulsion-based delivery system and storage time) by fixing the other factor at each specific level in order to determine significant differences in the particle size, gravitational stability, HTy content, FRAP assay, malondialdehyde content and instrumental colour. Means were separated by Tukey's HSD test ($\alpha = 0.05$). Pearson's correlation coefficient was used to study the relationships between FRAP assay and HTy content. A T-test was carried out to determine significant differences between the initial HTy content entrapped in the core of the DE and that at the end of the storage. $P \leqslant 0.05$ was deemed significant in all calculations.

3. Results and discussion

3.1. Microstructure and stability

Several primary emulsions (water-in-perilla oil, W_1/O) were produced as a first step for the production of DE-HTy-PO and GDE-HTy-PO. The same emulsification conditions were used in the second step to prepare DE-HTy-PO and GDE-HTy-PO as for SE-HTy-PO. The addition of gelatine and transglutaminase to the continuous phase of the DE caused the system to gel (GDE-HTy-PO) resulting in a structured (hydrogelled) emulsion which was solid at room temperature and thermally stable due to the formation of covalent bonds. Various gelling agents have been used to form emulsions gels but, to the best of our knowledge, the use of transglutaminase to form this cold-set GDE is completely novel and because of its viscoelastic properties offers interesting opportunities for the food industry as a fat replacer (Jiménez-Colmenero et al., 2015). SE-HTy-PO samples thus differed from DE-HTy-PO and GDE-HTy-PO mainly in their compartmentalization (characteristic of the emulsion type), whereas DE samples differed from GDE-HTy-PO in their external composition and viscoelastic properties. Regardless of these differences, all these emulsion-based delivery systems were characterized by the dispersion of perilla oil in a continuous aqueous phase (Fig. 1).

In the case of SE-HTy-PO, the dispersion of small oil droplets (the majority less than 5 μm) in a continuous aqueous phase was clearly observable (Fig. 1a) throughout the storage period (not shown). As in previous experiments (Cofrades et al., 2014), DE-HTy-PO had the characteristic compartmentalized structure (Fig. 1b and c) of this type of emulsions. In the larger droplets of DE-HTy-PO, very small water droplets were observed inside the lipid phase, which remained unchanged after 22 days of storage at 4 °C (Fig. 1c). GDE-HTy-PO was placed on slides for observation immediately after preparation and allowed to gel at 4 °C before microscopic observation the following day (Fig. 1d). This figure shows a continuous, covalently linked phase consisting of gelatine plus transglutaminase in which oil droplets seem to be closely packed. Inside the bigger droplets of GDE-HTy-PO it was also possible to see the typical DE compartmentalized structure in which small water droplets move freely inside the oil phase (See Supplementary Video). It was not possible to observe this system during storage due to evaporation of the outer aqueous phase of the prepared samples (on microscope slides), which disrupted the system. However, the appearance of the GDE-HTy-PO sample that was stored for analyses did not change over the storage period (not shown), and it therefore seems reasonable to assume that it would be as stable as DE-HTy-PO.

The particle size distributions of the SE-HTy-PO and DE-HTy-PO samples are plotted in Fig. 2. The monomodal distribution of DE-HTy-PO is consistent with previous studies dealing with other

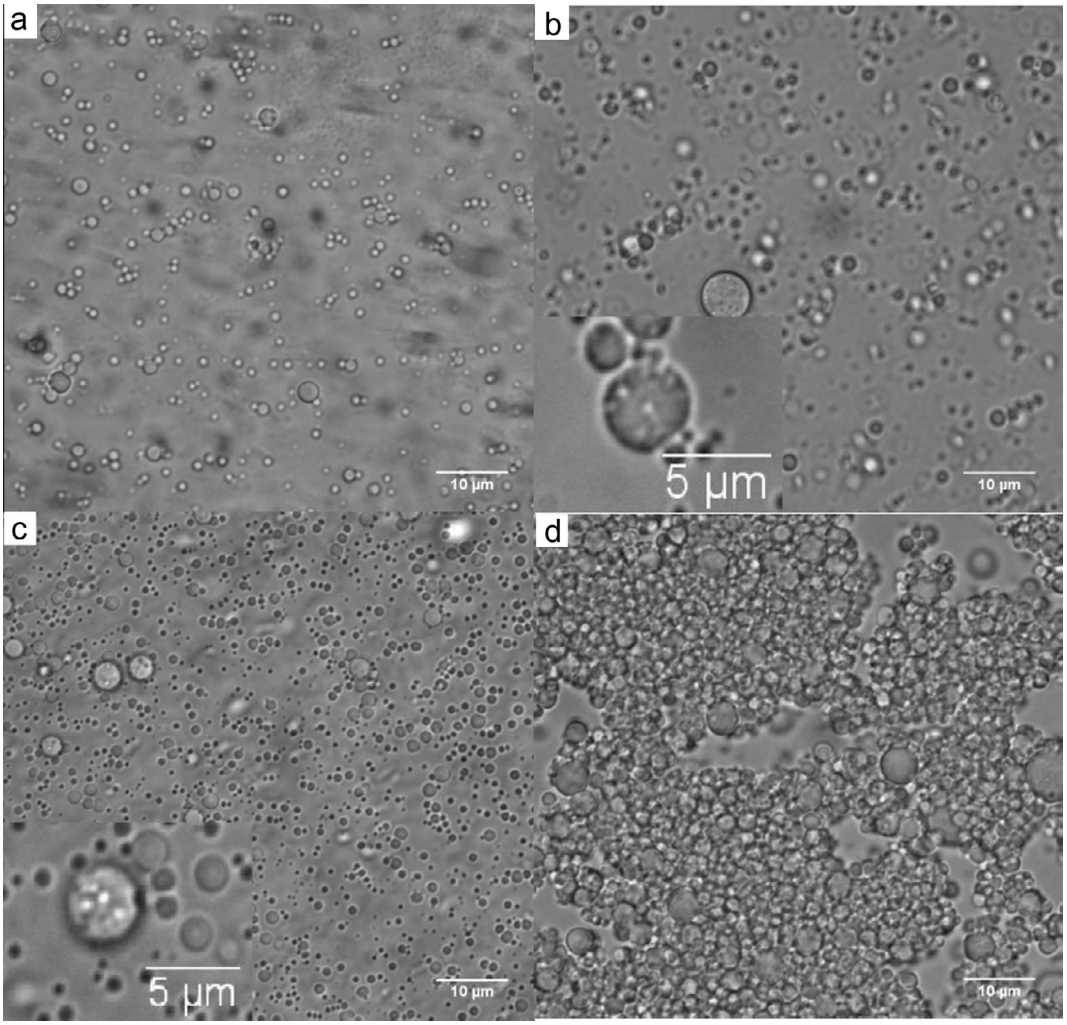

Fig. 1. Structures of the studied emulsions containing hydroxytyrosol and perilla oil stored at 4 °C: simple emulsion at day 1 (a); double emulsion at day 1 (b); double emulsion at day 22 (c) and gelled double emulsion at day 1 (d).

similar systems that differed in their lipid phases (Bou et al., 2014a, 2014b; Cofrades et al., 2014). Also, the similarities found between SE-HTy-PO and DE-HTy-PO can be accounted for the aforementioned common procedures used during the preparation of these emulsions. However, the latter were found to have a slightly narrower particle size distribution than SE-HTy-PO, especially at the beginning of storage. After 22 days of storage at 4 °C, the particle sizes of SE-HTy-PO and DE-HTy-PO were similar to the sizes at their respective initial times although size distributions were broader, especially in the case of SE-HTy-PO.

These observations are therefore consistent with the recorded droplet sizes (d_{43}) shown in Table 1. At the initial time, SE-HTy-PO and DE-HTy-PO showed similar droplet sizes. SE-HTy-PO remained unchanged during storage. At the end of storage, the droplet size of SE-HTy-PO was greater than that of DE-HTy-PO (Table 1 and Fig. 2). The trend in the droplet size of DE-HTy-PO

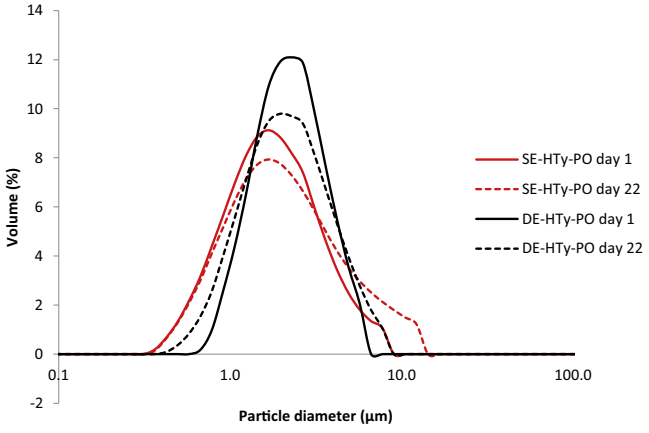

Fig. 2. Particle size distribution of simple and double emulsions after 1 day and 22 days of storage at 4 °C. See Table 1 for sample names.

Table 1
Particle size and gravitational stability of simple (SE-HTy-PO) and double (DE-HTy-PO) emulsions containing hydroxytyrosol and perilla oil.

	Day	SE-HTy-PO	DE-HTy-PO
Particle size (d_{43}; µm)	1	2.40 ± 0.19 x	2.22 ± 0.10 ab,x
	8	2.21 ± 0.31 x	2.03 ± 0.27 a,x
	15	2.53 ± 0.19 x	2.47 ± 0.14 b,x
	22	2.49 ± 0.15 x	2.29 ± 0.16 ab,y
Gravitational stability (%)	1	100 ± 0.00 a,x	100 ± 0.00 a,x
	8	98.97 ± 0.10 b,x	98.87 ± 0.15 b,y
	15	98.41 ± 0.17 c,x	97.35 ± 0.54 c,y
	22	97.58 ± 0.26 d,x	95.60 ± 1.00 d,y

Values are means ± standard deviation. Different letters in the same column (a, b, c, d) and in the same row (x, y, z) designate significant differences ($P \leqslant 0.05$).

was unclear, but in fact the values were similar at the beginning and at the end of storage. Overall, the droplet size of DE-HTy-PO was found to remain fairly constant up to 22 days of storage at 4 °C, which is enough time for a raw material to be used in the formulation of refrigerated meat and other food products. This is also consistent with findings of other authors dealing with different types of DE that had been stored for different periods at 4 °C or at room temperatures (Bonnet et al., 2009; Cofrades et al., 2014; Hattrem, Dille, Seternes, & Draget, 2014; Lutz, Aserin, Wicker, & Garti, 2009; Sapei, Naqvi, & Rousseau, 2012). Conversely, Hemar, Cheng, Oliver, Sanguansri, and Augustin (2010) reported small but significant particle size increases after 15 days of storage in DE stored at approximately 23 °C whereas Carrillo-Navas et al. (2012) reported a 25–35% increase in d_{43} after 5 months of storage at 4 °C. These storage periods and temperatures are therefore not comparable with the ones reported in this experiment.

The particle size of GDE-HTy-PO sample was not determined, as it cannot be diluted (after melting) due to the formation of a thermostable gel. However, Hattrem et al. (2014) compared DEs formulated with polysorbate 80 as a secondary emulsion emulsifier with others formulated with 20% and 30% gelatine as a hydrophilic emulsifier and gelling agent. These authors reported that the droplet size (d_{43}) of emulsions containing polysorbate 80 increased slightly during the first days and thereafter remained constant. Conversely, d_{43} remained unchanged after 90 days of storage at 20 °C in the emulsions formulated with gelatine. Therefore, it is reasonable to assume that the formation of the gel helped to maintain the droplet size of GDE-HTy-PO over the storage period.

The formation of emulsion gels offers interesting opportunities, such as fat replacement in food applications including meat products. GDE-HTy-PO remained solid throughout the storage without signs of phase separation and thus represents a clear advantage compared with other emulsion systems. In this connection, the phase separation at the end of storage was 2.4% in SE-HTy-PO and 4.4% in DE-HTy-PO (Table 1). This is a low level of phase separation given that the stability of DE-HTy-PO was comparable or even higher than reported in other studies dealing with different types of DEs (Bou et al., 2014b; Lutz et al., 2009; Sapei et al., 2012). According to Stokes' law, the phase separation depends on the viscosity of the emulsion, the difference in phase densities and the radii of the droplets. However, these parameters are relatively similar if we consider the data reported here and the preparation conditions for these systems. Because of their compartmentalization and increased interface/area ratio, DEs present considerable thermodynamic instability which is difficult to control. Therefore, those parameters that contribute to the phase separation of SEs are likely to have less influence on the overall destabilization of multiple emulsions that are less thermodynamically stable. In addition to this, the broader particle size distribution observed in SE-HTy-PO (Fig. 2) may also have been a factor in making creaming harder to visualize. Alternatively to the formation of GDE-HTy-PO, the stability of DE-HTy-PO can also be improved with the use of thickening agents (Dickinson, 2012).

3.2. HTy content, encapsulation efficiency and stability

The initial amount of HTy found in SE-HTy-PO and DE-HTy-PO samples after preparation is reported in Table 2. According to these results, the recovery yield for HTy was 99.5% and thus in line with reports for *trans*-resveratrol (95–98%) (Matos, Gutierrez, Coca, & Pazos, 2014). The HTy content found in GDE-HTy-PO was 292 mg/kg ± 5.2, similar to DE-HTy-PO (Table 2). A DE was prepared extemporaneously to form GDE-HTy-PO which started to gel in the process of cooling down to 4 °C. After 1 day of storage, the HTy content in GDE-HTy-PO had fallen to 215 mg/kg, which may be attributed in part to the temperature applied and the longer time needed to mix and dissolve the gelatine and transglutaminase. Nevertheless, no differences were found in HTy content of the studied systems. From day 1 to the end of storage, HTy decreased in SE-HTy-PO, DE-HTy-PO and GDE-HTy-PO by 8.6%, 26.9% and 24.2% respectively. Thus, SE-HTy-PO seemed to be much more stable given that no significant differences were found throughout storage (Table 2). Conversely, the HTy content decreased in more compartmentalized systems (DE-HTy-PO and GDE-HTy-PO). This increased susceptibility to HTy loss contributed to lower HTy contents in GDE-HTy-PO from day 8 to the end of storage when compared with the other emulsion systems. Despite that, the variability in the HTy content likely caused there were no differences between DE-HTy-PO and SE-HTy-PO after 22 days of storage. However, when regarding HTy losses in percentage it is reasonable to think that these systems with an increased surface area (GDE and DE) may behave similarly during storage. Therefore, the HTy loss that occurred during the gel formation (from day 0 to day 1), and the increased instability in more structured systems caused a lower content of HTy in GDE-HTy-PO at the end of the storage.

Table 2
Hydroxytyrosol content in simple (SE-HTy-PO), double (DE-HTy-PO) and gelled double (GDE-HTy-PO) emulsions.

	Day	SE-HTy-PO	DE-HTy-PO	GDE-HTy-PO
Hydroxytyrosol (mg/kg)	0	299 ± 2 a,x	298 ± 7 a,x	292 ± 5 a,x[1]
	1	267 ± 42 a,x	282 ± 5 ab,x	215 ± 21 b,x
	8	247 ± 39 a,y	253 ± 8 b,y	177 ± 21 b,c,x
	15	251 ± 4 a,y	222 ± 9 c,y	179 ± 12 b,c,x
	22	244 ± 17 a,y	206 ± 19 c,y	163 ± 2 c,x

Values are means ± standard deviation. Different letters in the same column (a, b, c, d) and in the same row (x, y, z) designate significant differences ($P \leqslant 0.05$).
[1] Before adding gelatine and transglutaminase.

The encapsulation of HTy in more compartmentalized systems, however, may offer some advantages compared to SE-HTy-PO in that HTy loss can be prevented by such encapsulation. Note that HTy encapsulation efficiency in DE-HTy-PO was 55% (Supplementary Figure). This efficiency was not improved by the addition of caseinate or gelatine in the inner water phase (data not shown). The encapsulation and release of encapsulated compounds depends on various factors including the composition and the technology used to prepare the DE as well as the nature of the encapsulated compound (Dickinson, 2011). All these factors explain the different encapsulation efficiencies for the limited number of phenolic compounds that have been encapsulated in various DEs. For instance, encapsulation efficiency for catechin has been reported to be as high as 97% (Aditya et al., 2015), whereas for *trans*-resveratrol it has been reported to range from 25% up to 40% (Matos et al., 2014). These last authors reported higher efficiencies with increased amounts of hydrophilic and hydrophobic emulsifiers (Tween 20 and PGPR respectively). In our case, the formation of a thermally stable GDE-HTy-PO made it difficult to determine whether HTy encapsulation had a synergistic effect upon the addition of gelatine and transglutaminase. However, it is reasonable to assume that HTy encapsulation efficiency was similar in DE-HTy-PO and GDE-HTy-PO, as in our case the gelation process is an additional step that occurred immediately after formation of the DE.

HTy is highly water-soluble, but because of its partition coefficient ($K_p = 0.01$) it is also partially dissolved in the oil phase (Rodis, Karathanos, & Mantzavinou, 2002). Therefore, HTy is expected to transfer from the inner to the outer aqueous phases through the lipid phase during storage and regardless of coalescence and breakdown mechanisms. Surprisingly, the encapsulation of HTy was increased from 55% to 72% ($P = 0.004$) at the end of the storage (Supplementary Figure). It is important to note that the amount

of HTy remaining within the core of the emulsion (W_1/O) stayed fairly constant throughout storage (168–149 mg/kg) and the calculation of encapsulation stability takes into account the total amount of HTy that is present in each storage period. Therefore, the main reason for this encapsulation stability increase was presumably the preferential loss of the HTy located in the outer aqueous phase. Unlike other phenolics that interact strongly with proteins, HTy has a weak affinity for typical food proteins including caseinate and gelatine (Pripp, Vreeker, & van Duynhoven, 2005). Therefore, the fact that HTy loss was smaller in GDE-HTy-PO than in DE-HTy-PO (24.2% vs 26.9%) from day 1 to day 22 supports the hypothesis that the gel network minimized HTy loss during storage by several means including physical stabilization of the emulsion, reduction of HTy diffusion to the outer phase and immobilization of pro-oxidant compounds such as transition metals.

Table 3
Ferric ion reducing antioxidant power (FRAP), malondialdehyde content and instrumental colour (L^*, a^*, b^*) in simple (SE-HTy-PO), double (DE-HTy-PO) and gelled double (GDE-HTy-PO) emulsions.

		Day	SE-HTy-PO	DE-HTy-PO	GDE-HTy-PO
FRAP (μM)		1	3498 ± 368 a,y	3733 ± 90 a,y	2655 ± 256 a,x
		8	3454 ± 506 a,y	3521 ± 95 a,b,y	2692 ± 180 a,x
		15	3559 ± 90 a,y	3117 ± 82 c,y	2352 ± 351 a,x
		22	3289 ± 261 a,y	3136 ± 256 b,c,y	2319 ± 91 a,x
Malondialdehyde (nmol/g)		1	1.4 ± 0.51 a,x	1.3 ± 0.60 a,x	3.9 ± 0.74 a,y
		8	0.9 ± 0.26 a,x	0.4 ± 0.16 b,x	2.8 ± 0.63 a,y
		15	0.9 ± 0.75 a,x	0.3 ± 0.04 b,x	2.9 ± 65 a,y
		22	0.9 ± 0.56 a,x	0.3 ± 0.07 b,x	2.5 ± 0.11 a,y
Colour	L^*	1	87.1 ± 1.1 a,y	84.9 ± 0.8 a,x	84.3 ± 0.8 a,x
		8	76.7 ± 0.9 b,y	72.8 ± 1.8 b,x	80.2 ± 0.5 b,z
		15	70.7 ± 1.9 c,x	70.9 ± 4.5 b,c,x	79.0 ± 0.5 c,y
		22	71.1 ± 1.4 c,y	69.2 ± 2.2 c,x	78.3 ± 0.5 d,z
	a^*	1	2.5 ± 0.2 a,z	3.2 ± 0.5 y	0.5 ± 0.1 a,x
		8	3.5 ± 0.4 c,z	3.0 ± 0.4 y	0.8 ± 0.1 c,x
		15	3.5 ± 0.4 c,z	3.3 ± 0.4 y	0.8 ± 0.1 c,x
		22	2.9 ± 0.3 b,y	3.0 ± 0.6 y	0.7 ± 0.1 b,x
	b^*	1	4.8 ± 0.2 a,x	5.8 ± 0.6 a,y	4.4 ± 0.2 a,x
		8	12.0 ± 0.5 b,y	13.3 ± 1.2 b,z	6.8 ± 0.6 b,x
		15	14.8 ± 0.3 c,y	15.9 ± 4.2 c,y	7.8 ± 0.3 c,x
		22	16.6 ± 1.6 d,y	16.8 ± 1.0 c,y	8.6 ± 0.4 d,x

Values are means ± standard deviation. Different letters in the same column (a, b, c, d) and in the same row (x, y, z) designate significant differences ($P \leqslant 0.05$).

3.3. Oxidative Stability: antioxidant capacity, malondialdehyde content and colour

The systems considered here can serve as n-3 PUFA delivery systems in different foods since they are designed to contain over 30% of perilla oil. This oil is of special interest in that it is one of the richest sources (over 60 g/100 g) of α-linolenic acid, with demonstrated preventive effects on atherosclerosis and chemically-induced cancer, and it also has beneficial effects on immune and mental functions (Jo, Kim, Lee, Kim, & Song, 2013). These systems contained over 18 g/100 g of α-linolenic acid, which is very prone to oxidation. Therefore, in addition to HTy content (Section 3.2.), antioxidant capacity, malondialdehyde content and colour were determined to assess the oxidative stability of these emulsion-based delivery systems (Table 3). At the initial time there were no differences in malondialdehyde content between SE-HTy-PO and DE-HTy-PO, whereas higher levels were recorded in GDE-HTy-PO. The initial hydroperoxide content of DE-HTy-PO and GDE-HTy-PO was 0.06 ± 0.002 and 0.16 ± 0.01 mmol CHP/kg, respectively. These results are therefore in line with the increased MDA content in the gelled system and attributed to the longer and harsher preparation conditions.

The levels of malondialdehyde in SE-HTy-PO and GDE-HTy-PO remained unchanged during storage, but they decreased in DE-HTy-PO. It is worth to mention that these results are in agreement with TBARS values (data not shown) and also with the fact that free and bound MDA are measured by means of the reported HPLC method (Grotto et al., 2009). Although MDA values decreased in DE-HTy-PO after 8 days of storage, this change (0.9 nmol/g) and the amounts found can be considered minimal if compared with other secondary oxidation decreases reported during the storage of various emulsions (Decker, Alamed, & Castro, 2010a). Besides, oxidation levels remained stable from day 8–22. Therefore, DE-HTy-PO and the other emulsions (SE-HTy-PO and GDE-HTy-PO) can be considered as stable during the whole storage. Indeed, HTy has been proved to be effective in minimizing oxidation in meat systems when this was incorporated within a similar DE (Cofrades et al., 2014). However, in order to confirm that the oxidative status maintenance throughout the storage period we decided to compare during 50 days of storage a GDE containing HTy (W_1 with 100 mg/kg) with another elaborated without this antioxidant (Fig. 3). In this experiment it can be observed that TBARS and lipid

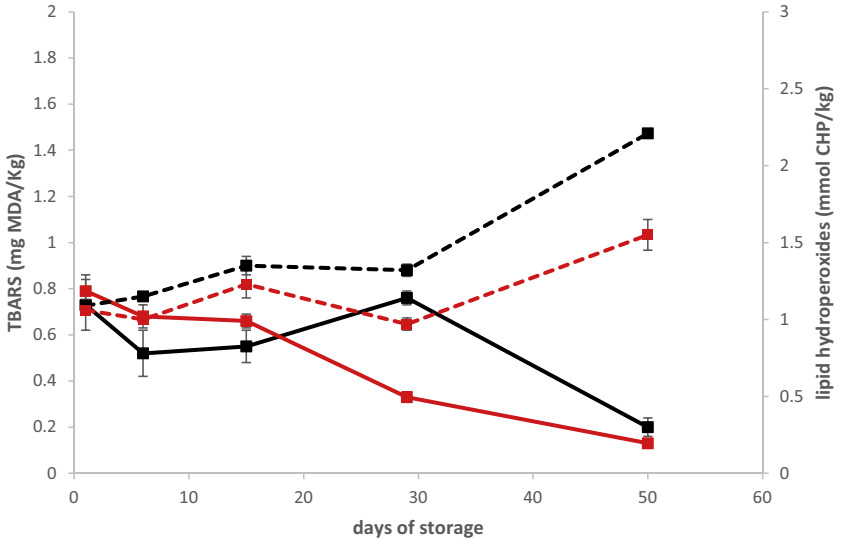

Fig. 3. Evolution of lipid hydroperoxides (segmented line) and secondary oxidation values measured by means of TBARS method (continuous line) in gelled double emulsions elaborated without hydroxytyrosol (in black) or using an inner aqueous phase containing 100 mg/kg (in red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hydroperoxides values of both samples remained almost unchanged until approximately 30 days. Later on TBARS values decreased in both GDE and, conversely, the content in lipid hydroperoxides increased although more markedly in the GDE without HTy. Overall, these results suggest that these systems are stable and the presence of HTy may protect from oxidation. Regarding these results, it may be advisable to monitor primary oxidation and other secondary oxidation products (e.g. hexanal and propanal) in future experiments over longer storage periods to gain a better understanding of the oxidation in these systems. Despite that, in this experiment these systems seem to be stable up to 22 days and protected by the presence of HTy. Accordingly, the observed differences in lipid oxidation and, more noticeably, in the HTy content between these systems may then be related to several phenomena mainly associated with their structural characteristics (increased surface area) and processing conditions (use of high temperatures for longer times).

The overall antioxidant capacity seemed to be mainly determined by the amount of HTy present in the different emulsions (Tables 2 and 3) as they correlated closely ($r = 0.83$). For instance, the lowest initial antioxidant capacity was recorded in GDE-HTy-PO (Table 3) which, as discussed previously, may be attributed to HTy loss during preparation. On the other hand, the presence of gelatine and transglutaminase in the GDE-HTy-PO could affect lipid oxidation. Sato, Moraes, and Cunha (2014) reported a reduction of oil oxidation in gelled emulsions in the presence of gelatine. Gelatine has the ability to adsorb on the oil/water interface and may act as a physical barrier, separating the lipid substrates from the pro-oxidants present in the aqueous phase (McClements & Decker, 2000). However, in our experimental conditions, GDE-HTy-PO had the highest initial oxidation level and therefore no such protective effect of gelatine was observed, or at least it was not enough to overcome the effect of other components such as transglutaminase. Delgado-Pando et al. (2011) reported that lipid oxidation was greater when transglutaminase was included in an oil-in-water emulsion. Additionally, the increase of oxidation values in GDE-HTy-PO may be attributed to the use of relatively high temperatures and extended times for complete solubilisation of gelatine during the preparation of GDE-HTy-PO, as opposed to the preparation conditions used to form SE-HTy-PO and DE-HTy-PO, which did not require additional heating. Cofrades et al. (2014) reported that some degree of oxidation occurs during the production of DEs, stressing the desirability of protecting the lipid phase with antioxidant-enriched DEs. This can be prevented by the addition of HTy, although processing conditions need to be optimized for minimum loss of HTy and maximum protection of n-3 fatty acids.

The largest decrease in antioxidant capacity during storage was observed in DE-HTy-PO (Table 3), presumably associated with the increased susceptibility to oxidation in more compartmentalized systems as noted above. Oxidation has been reported to be mainly initiated at the interface by interaction between pro-oxidant factors and the lipids to be oxidized (Decker et al., 2010b; McClements & Decker, 2000). Oxygen and edible oil react more promptly when the oil droplet size is small or has a high surface to volume ratio, as in the case of emulsions (Sato et al., 2014). Therefore, the higher HTy loss and lower antioxidant capacity found in DE-HTy-PO and GDE-HTy-PO may be related to the increase in the interface area/volume ratio of these emulsions. As in the present experiment, Cofrades et al. (2014) found that TBARS levels in DEs with HTy decreased during storage. These authors reported that oxidation levels over storage were lower than in the same emulsions without HTy, demonstrating the antioxidative effect of HTy. The location and slow release of HTy reservoir into the lipid phase may be responsible for inhibiting oxidation during storage. The maintenance (or minimal decrease) of malondialdehyde during storage with FRAP values and HTy loss, suggesting that the latter serves to protect other food components. The results evidence the antioxidant potency of HTy, which maintained high levels after 22 days of storage (Table 2), protecting n-3 fatty acids against oxidation as shown by the malondialdehyde content. Therefore, these delivery systems containing HTy can help to prevent oxidation when used as functional ingredients.

Colour is an important characteristic of delivery systems since it may influence the final appearance of the food product in which they are used and hence determine its overall acceptability. At the outset, all the systems containing n-3 fatty acids and HTy presented a similar colour. In spite of this, the initial L^*, a^* and b^* values of these emulsions were found to be affected by the type of system (Table 3). This means that the specific composition, preparation conditions and oxidation status may have contributed to the final colour of the emulsions. Lightness was found to decrease under storage, whereas yellowness did the opposite. However, there was no clear trend in the case of redness. Therefore, it seems reasonable to suppose that the onset of oxidation associated with HTy loss and the further transformation into brownish quinones contributed to these colour changes and should be taken into account when using these delivery systems in real food samples.

4. Conclusions

The simultaneous incorporation of n-3 fatty acids and HTy in foods can be achieved by means of several types of emulsions possessing different characteristics. The development of more complex systems may offer some interesting advantages such as encapsulation of bioactive compounds (e.g. DEs) and/or desired plastic properties (e.g. GDEs). From a physical point of view, all systems were shown to be stable and the addition of 300 mg/kg HTy efficiently protected n-3 fatty acid-enriched emulsions from oxidation. However, antioxidant capacity diminished with storage, and there were also colour changes possibly related to the onset of oxidation. HTy loss mainly occurred during preparation of GDE-HTy-PO and during storage in all of them. This antioxidant loss progressed faster in more compartmentalized systems (DE-HTy-PO and GDE-HTy-PO), which was attributed to their increased surface area. However, the formation of GDE-HTy-PO may minimize this loss during storage when compared with DE-HTy-PO. Therefore, when using DEs the encapsulation of labile compounds needs to be considered and adapted to specific technological and/or nutritional requirements.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.06.005.

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