Emulsion gels containing n-3 fatty acids and condensed tannins as functional fat replacers for meat products: physicochemical properties and stability

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ABSTRACT

Cold set emulsion gels offer interesting possibilities as functional ingredients. Thus, different oil-in-water emulsions rich in n-3 fatty acids were gelled upon the addition of gelatin, k-carrageenan and transglutaminase in combination or not of condensed tannins. This study examines the effect of the protein used to stabilize the emulsions (sodium caseinate, whey protein and isolated soy protein) on instrumental color, content in extractable and non-extractable proanthocyanidins, syneresis, antioxidant capacity and oxidative stability of these emulsion gels after 28 days of storage at 4 °C. The color of the emulsion gels was affected by the type of emulsifier and the addition of condensed tannins. The existence of complex interaction between proteins and condensed tannins explained the different content in non-extractable tannins. The phenolic content explained the increased antioxidant capacity and oxidative status of those emulsions gels formulated with condensed tannins. Overall, the studied delivery systems showed excellent binding properties and a minimal progression of oxidation which guarantees their use as functional fat replacers.

Keywords: Gelled emulsion, cold set gels, fat replacers, condensed tannins, n-3 fatty acids

1. INTRODUCTION

Reformulation is one of the most important approaches for improving the fat content of meat products and developing healthy and functional foods (Jimenez-Colmenero, 2007). In this regard, the consumption and development of food products enriched with n-3 fatty acids is of particular interest because of the reported beneficial health effects, mainly relating to cardiovascular and inflammatory diseases (Ruxton, Reed, Simpson, & Millington, 2004; Simopoulos, 2006). However, these unsaturated lipid materials have different physicochemical characteristics from fats which are normally used in the food industry. Therefore, the removal or substitution by liquid lipids (oils) in the reformulated product may have a negative effect on the desired quality attributes as in the case of meat products.

Recently, novel proposals for oil stabilization and structuring have been reported to develop fat alternatives which can be used to improve the quality of the reformulated systems (Jiménez-Colmenero et al., 2015). These strategies are aimed to mimic a plastic fat and thus retain solid-like properties while possessing a healthier fatty acid profile. In this context, the formation of structured emulsions such as gelled emulsions (GE; or emulsion hydrogels) offer interesting possibilities as fat replacers. Among different possibilities, the initial stage in emulsion gel formulation typically involves the production of a protein stabilized liquid oil-in-water (O/W) emulsion. By using thermal, enzymatic or chemical means a solid-like emulsion gel may be generated from a stable liquid-like emulsion by gelling the continuous aqueous phase. The gelation process depends, to a great extent, on the nature of the system (Jiménez-Colmenero et al., 2015). However, those gels formed by non-thermal treatments are referred to as cold set gels and have shown to be particularly well suited to deliver heat-labile bioactives and nutraceuticals (Jones & McClements, 2010; Yang, Liu, & Tang, 2013; Zeeb et al., 2013).

The employment of enzymes such as microbial transglutaminase (MTG) to cross-link protein components and thus form thermally stable emulsion gels gained great interest when dealing with labile ingredients such as n-3 fatty acids (Flaiz et al., 2016; Herrero,

Carmona, Pintado, Jimenez-Colmenero, & Ruiz-Capillas, 2011; Yang et al., 2013). This enzyme induces covalent cross-linking by the acyl transfer between glutamine and lysine residues in proteins (Motoki & Seguro, 1998; Yang et al., 2013). In comparison to heat-induced gels, those emulsion gels formed by means of MTG not only minimize lipid oxidation of n-3 fatty acids during their elaboration but they may also offer additional protection in food products (Flaiz et al., 2016). In this connection, various biopolymers such as gelatine, carrageenans have also been used to elaborate different types of cold-set emulsion-filled gels (Poyato, Ansorena, Berasategi, Navarro-Blasco, & Astiasaran, 2014; Sala, van Vliet, Stuart, van de Velde, & van Aken, 2009).

Lipid oxidation not only causes nutritional loss and rancidity but also constitutes a potential hazard in that several of the compounds thus formed have been associated with neurodegenerative and cardiovascular diseases (Esterbauer, Wag, & Puhl, 1993; Perluigi, Coccia, & Butterfield, 2012). In this context, the antioxidant addition represents a suitable strategy to tackle this problem especially if it involves the use of natural compounds and extracts as they are widely accepted by consumers (Ahn, Grun, & Fernando, 2002; Decker, Elias, & McClements, 2010). In this context, the use of Exxenterol, a denatured carob dietary fibre very rich in condensed tannins (CT) in the form of oligomeric proanthocyanidin complexes, demonstrated its antioxidant efficacy in frying oils (Sanchez-Muniz et al., 2007) and meat systems (Bastida et al., 2009). Moreover, diets rich in dietary fibre have been associated to a decreased risk of colon cancer and cardiovascular disease (American Dietetic Association, 2008; Bingham et al., 2003). In this connection, the insoluble dietary fibre from carob pod has shown hypocholesterolemic properties in animal (Perez-Olleros, Garcia-Cuevas, Ruiz-Roso, & Requejo, 1999; Wursch, 1979) and clinical trials (Ruiz-Roso, Quintela, de la Fuente, Haya, & Perez-Olleros, 2010; Zunft et al., 2003).

Therefore, this study is aimed at examining the potential of several food-grade O/W gels (GE) to be used as healthier fat replacers. For the preparation of the different emulsions a lipid phase rich in n-3 fatty acids and three commonly used emulsifiers, namely sodium caseinate (SC), whey protein isolate (WPI) and isolated soy protein (ISP) were employed with and without the addition of CT. These emulsions were thereafter induced to form cold-set gels designed to improve the lipid content quantitatively and qualitatively for

their use in various food applications such as meat products. The addition of a natural extract rich in polyphenols in the formulation of these emulsions was aimed to not only protect from oxidation but also because of their reported beneficial health effects. Accordingly, these GE were characterized and their oxidative stability was evaluated during their storage at 4 °C.

2. MATERIAL AND METHODS:

2.1. Materials and reagents

Extra virgin olive oil (Carbonell Virgen Extra, SOS Cuétara SA; Madrid, Spain), linseed oil (Natursoy, Alimentos Ecológicos; Castellterçol, Spain) and fish oil (Omevital 18/12 TG Gold; Cognis GmbH; Illertissen, Germany) were used as lipid phases in emulsion preparations. Sodium caseinate (Excellion EM 6, *FrieslandCampina* DMV; Veghel, the Neteherlands), whey protein isolate (Provon 295, Glanbia Nutritionals; Kilkenny, Ireland) and isolated soy protein from (Wilpro G-300, Wilmar Group; Qinhuangdao, China) were used as emulsifiers and hereafter simple referred as SC, WPI and ISP, respectively. Condensed tannins (Exxenterol®; CT) extracted from carob pod were obtained from Biosearch SA (Granada, Spain). Bovine gelatine (200-220 bloom) was from Manuel Riesgo, S.A. (Madrid, Spain), Texturalia k-carrageenan was from Trades S.A. (Barcelona, Spain) and Activa GS microbial transglutaminase (MTG) was from Ajinomoto (Tokyo, Japan).

Methanol, chloroform stabilized with ethanol, 1-butanol, trichloroacetic acid, ammonium thiocyanate, ammonium sulphate, sodium chloride, ferrous sulphate heptahydrate, barium chloride dihydrate, sodium carbonate, hydrochloric acid, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), sodium azide and hexane were from Panreac Quimica, SA (Barcelona, Spain). The 2-thiobarbituric acid reagent was from (Merck KGaA, Madrid, Spain) whereas 1,1,3,3-tetraethoxypropane (TEP) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), butylated

hydroxytoluene (BHT), gallic acid and Folin-Ciocalteau reagent were from Sigma-Aldrich (Madrid, Spain).

2.2. Formulation and preparation of O/W emulsion gels

The composition of the emulsions and their process of elaboration was optimized in previous tests to obtain various gelled emulsions with the desired physicochemical characteristics. In this study, six different types of oil-in-water (O/W) emulsion gel samples were prepared according to the formulations described in Table 1. These O/W emulsions were prepared by mixing six different aqueous phases containing SC, WPI or ISP as emulsifiers (added at 2, 6 and 3 g/100 g, respectively) with and without the presence of CT. In order to obtain a healthier lipid formulation in line with health recommendations, a specific lipid phase consisting in a combination of different edible oils was designed (Delgado-Pando, Cofrades, Ruiz-Capillas, Solas, & Jimenez-Colmenero, 2010). Accordingly, these emulsions were elaborated with olive oil, linseed oil and fish oil added at 44.39, 37.87 and 17.74 g/100 g oil, respectively.

Coarse emulsions were elaborated by the dropwise addition of the lipid phase (50 g/100 g) into a food processor (Thermomix, Vorwek, Germany) containing the different aqueous phases (50 g/100 g) while mixing at 3250 rpm for 20 min at room temperature. Those coarse emulsions prepared with SC and WPI were passed once through a high-pressure homogenizer (GEA Niro Soavi MODEL Panda Plus 2000, Parma, Italy) at 7/55 MPa (first-stage pressure/second-stage pressure) to obtain fine emulsions. Those emulsions prepared with SC and WPI are referred as SC- and WPI-emulsions. The coarse emulsion prepared with ISP was not homogenized as their counterparts as its process lead to an increased viscosity which makes difficult its further processing. This latter emulsion is hereafter referred as ISP-emulsion. The same elaboration procedure was used to elaborate those emulsions with CT, which were added after each emulsion preparation (3.9 g/100 g emulsion) and subsequently mixed. These emulsions are hereafter referred as SC-Ex, WPI-Ex and ISP-Ex emulsions.

To structure the different emulsions one twentieth volumes of water were separately heated to 80 °C in a food processor at 1625 rpm. Then, κ-carrageenan was added at 0.3 g/100 g of emulsion and stirred until its complete dissolution followed by the addition of bovine gelatine at 0.5g/100g of emulsion. Once solubilised, this solution with hydrocolloids was mixed with the different O/W emulsions while stirring at 1625 rpm in the food processor set at 37 °C. Similarly, one and half grams of microbial transglutaminase (MTG) per 100 g of emulsion were added in one tenth volumes of water. Once solubilized, they were immediately mixed with their respective emulsions with hydrocolloids until its complete homogenization. Subsequently, aliquots of 80 ml were rapidly filled into 100 ml tubes and thereafter stored at 4 °C. Under these conditions, two gelled emulsions with SC (GE-SC-C and GE-SC-Ex), two gelled emulsions with WPI (GE-WPI-C and GE-WPI-Ex) and two gelled emulsions with ISP (GE-ISP-C and GE-ISP-Ex) were formed within 24 h.

2.3. Instrumental colour and pH

CIE-Lab tristimulus values were analysed using a spectrophotometer 3500d (Konica Minolta Business Technologies, Tokyo, Japan). The instrument was set for standard illuminant D-65 and a 2 ° observer angle and standardized using white and black standards. Three determinations were performed from each formulation.

The pH was determined twice on 10 g of sample homogenate in 100 ml of deionized water using an Orion Research 720A pH meter (Instrumentación Analítica SA, Madrid, Spain).

2.4. Extraction and determination of proanthocyanidins

The amount of condensed tannins in samples included the extractable proanthocyanidins (EPA) and the non-extractable proanthocyanidins (NEPA) fractions which were determined in duplicate.

The extraction of EPA is based on the method reported elsewhere (Pérez-Jiménez, Arranz, & Saura-Calixto, 2009). In brief, 1 g of emulsion was homogenized with 20 ml of the extraction solution consisting of 2N HCl (pH = 2) dissolved in methanol/water (50:50, v/v). Samples were placed for 1 hour at 180 rpm in an orbital shaker (Ika S.A., Staufen, Germany) and subsequently centrifuged at 20,000 *g* for 50 min at 4 °C (Sorvall evolution RC superspeed, Thermo Scientic, USA) to obtain the methanol-water soluble phase from an insoluble residue. This supernatant contains part of the EPA and was filtered through Whatman No. 1 filter paper. The residue was re-extracted with a acetone/water solution (70:30, v/v) and after stirring samples were centrifuged again. The obtained supernatant was mixed with the latter and transferred to volumetric flasks and filled up with a 50:50 (v/v) mixture of the acidic methanol/water and acetone/water extraction solutions. This combination of extracts corresponds to the total amount of EPA.

The Folin-Ciocalteu method was employed for the determination of EPA (López-López et al., 2009). A 5 µl sample aliquot of the EPA extract reacted with 5 µl of the Folin-Ciocalteu reagent, 100 µl of sodium carbonate and 140 µl of milliQ water. The absorbance at 750 nm was measured for 1 h every 10 min in the PowerWave XS spectrophotometer (BioTek, Winooski, VT, USA). A calibration curve was prepared with gallic acid ranging from 0 to 0.5 mg/ml. Results were expressed as mg of gallic acid eqv/ g product.

The EPA insoluble residue was used for the determination of NEPA according to a previously procedure described (Zurita, Diaz-Rubio, & Saura-Calixto, 2012). Ten ml of butanol/HCl (97.5:2.5, v/v) containing 0.7 g of $FeCl_3$ were added to this residue and heated at 100 °C for 60 min. Then, samples were cooled to room temperature and centrifuged at 509 g for 15 minutes and the supernatant was colleted. The residue was re-extracted several times with 5 ml of above solution and all the volumes were combined and filled up to 25 ml. The absorbance was measured at 555 and 450 nm by means of the UV-Vis spectophotometer. Standard curves were obtained for carob pod tannins concentrate. The results were expressed as mg of NEPA/ g product.

2.5. Physical stability of the gelled emulsion

Syneresis was determined in duplicate as a measure of emulsion gel stability. Fat and water losses were determined by measuring the released exudate during the storage of GE at 4 °C. Briefly, tubes containing the GE were opened and then were left to stand upside down for 15 min to release the exuded fat and water onto a plate. Total loss was expressed as % of initial sample weight.

2.6. Antioxidant capacity

Antioxidant activity was determined in the NEPA extracts in duplicate using an automated photochemiluminescent system (Photochem©, Analytik Jena Model AG; Analytic Jena USA, The Woodlands, TX, USA), which measures the capacity to quench free radicals (Popov & Lewin, 1996). Briefly, 20 µL of the extract containing NEPA were added to reagent kits supplied by the manufacturer and the automated system measured the total antioxidant capacity. Trolox was used as a standard, and results were expressed in Trolox Equivalents (mmol TE/g sample).

2.7. Lipid extracts and oxidation

The lipid fraction of the gelled emulsions was extracted as in the Bligh and Dyer method with minor modifications (Bligh & Dyer, 1959). In brief, 6 g of sample were homogenised with a mortar and pestle with 1 ml of water and 14 ml of methanol, then 7 ml of chloroform were added and mixed again. This latter step was repeated with and, subsequently, 7 ml of water were added and mixed. The homogenate was then filtered by means of a vacuum flask and the filtrate was stored in the dark at 4 °C overnight. Then, the upper phase was discarded and the organic phase was evaporated at 40 °C by means of a rotary evaporator (Büchi; Flavil, Switzerland). The lipid extract was aliquoted and immediately stored at -80 °C with the exception of those aliquots that were destined to the determination of the hydroperoxide content (0.2 g) which were previously dissolved in 10 ml of chloroform/methanol (1:1, v/v) and thereafter stored at -80 °C.

2.7.1. Content in Lipid hydroperoxides

The content in lipid hydroperoxides was measured in duplicate as described elsewhere with minor modifications (Matalanis, Decker, & McClements, 2012). Briefly, 0.2 ml of the latter chloroform/methanol solution is mixed with 2.8 ml of methanol/1-butanol (2:1, v/v). Following, 15 µl of 3.94 M ammonium thiocyanate and 15 µl of ferrous iron solution (prepared by reacting equal volumes of 0.132 M barium chloride and 0.144 M ferrous sulfate) were added to this mixture (3 ml). This was vortexed and allowed to react for 20 min at room temperature in the dark before the absorbance of the sample was measured at 510 nm using a Shimadzu UV-1800 spectrophotometer (Shimadzu Inc., Kyoto, Japan). The concentration of hydroperoxides was determined based on a standard curve of cumene hydroperoxide (0–20 µM). Results were expressed as mmol hydroperoxides/kg oil.

2.7.2. Determination of thiobarbituric acid reactive substances (TBARS)

TBARS values were determined in duplicate by means of the thiobarbituric acid reactive substances (TBARS) method as reported elsewhere (Poyato, Astiasaran, Barriuso, & Ansorena, 2015). Briefly, 0.25 g of lipid extract obtained from gelled emulsions were mixed with 55 ml of water, 20 ml of BHT (1 g/100 ml hexane) and 2 ml of a TBARS reagent solution consisting in trichloroacetic acid (15 g/100 ml), 2-thiobarbituric acid (0.375 g/ 100 ml) and hydrochloric acid (0.25 g/100 g). The mixture was placed in a boiling water bath for 15 min exactly. Samples were then cooled by immersing in ice. Then, 1 ml of 4 M ammonium sulphate were added to the mixture and vortexed for 1 min. Subsequently, samples were centrifuged at room temperature at 662 *g* for 30 min at 10 °C (Multifuge 3 LR, Heraeus, DJB Labcare Ltd.; Buckinghamshire, UK). The absorbance of the lower phase was measured at 532 nm. Concentrations were determined from a malondialdehyde (MDA) standard curve produced from TEP. Results were expressed in mg MDA/kg oil.

2.8. Statistical analysis

The experiment was replicated. A MANOVA was used to determine the effect of the main factors the GE formulation (i.e. type of emulsifier and the addition of condensed tannins) and their interaction on emulsion gel characteristics and oxidative stability. In addition, series of one-way ANOVAs were performed for the factors: GE formulation and storage time by selecting the other factor at each specific level. Least-squares means and means were separated by Tukey-HSD test when significant ($p<0.05$). Statistical analysis was carried out using IBM SPSS statics 22 software (IBM SPSS Inc., Chicago, IL).

3. RESULTS AND DISCUSSION

All GE formulated without CT presented a whitish solid-like appearance that resembles that of fat and thus can be used as a fat replacer in a range of foods including meat products. The aspect of those GE with CT was more greyish as a result of this ingredient addition (Figure 1). Despite that, the overall appearance of those gelled emulsions with CT is, depending on the amount and the product to be applied, still suitable for its intended use.

3.1. Effect of the emulsifier and the addition of condensed tannins

A MANOVA was carried out to gain a better understanding of the overall effects of the type of emulsifier and the addition of CT on the physicochemical characteristics of the different GE. Table 2 shows the color of the different GE and the fact that the addition of CT decreased ($P < 0.01$) lightness and yellowness values whereas redness was increased (Table 2). These effects on the different GE can be attributed to the intrinsic color of the ingredient. This finding is in agreement with previous studies in which different lipid phases and bioactive compounds (e.g. hydroxytyrosol) incorporated in the

inner aqueous phase of DE and GE influenced on the color of the studied delivery systems (Bou, Cofrades, & Jimenez-Colmenero, 2014b).

Likewise, the color of the different GE was affected by the type of emulsifier (Table 2). Lightness least-squares means for those GE with ISP, WPI and SC were 63.8, 74.2 and 76.3, respectively, and found to be all different between them ($P < 0.001$). The opposite trend was observed for redness and yellowness least-squares means (SC < WPI < ISP). In this regard, Flaiz et al. (2016) reported that the addition of gelling agents into DE to form GE caused changes in color. In agreement with this, Delgado-Pando et al. (2010) reported color differences in oil-in-water emulsions with caseinate or ISP. Cofrades et al. (2013) reported that redness was reduced in those meat systems in which double emulsions with caseinate were added whereas it remained unchanged when they involved the addition of double emulsions stabilized with whey protein. These aspects should be taken into account provided that the final color of the system can be influenced by the addition of different biopolymers in the external aqueous phase.

The pH of the extract rich in CT was 4.6 thus explaining the decreased pH in those GE containing this ingredient when compared with their homologues. Likewise, the employment of different proteins to form GE caused changes in the pH of these systems (Table 3). In this regard, GE-WPI-C recorded pH values of 7.6 after preparation whereas those GE with SC and ISP (GE-SC-C and GE-ISP-C) recorded higher values which, in turn, were different between them. This pH range is suitable for its application as ingredient in meat product formulations with realistic inclusion levels ranging between 10-15% (Cofrades et al., 2013).

Control samples showed a low EPA content (Table 3), which may come either from phenolics present in the oils employed to form the emulsions (Xiang et al., 2017) or from the interferences in the method that amino acids may cause (Prior, Wu, & Schaich, 2005). As expected, the addition of CT caused a significant increase in EPA and NEPA contents when compared with their counterparts. Regarding EPA, their overall mean content was unaffected by the proteins employed to elaborate the emulsions. In this regard it is worth noting that, in all samples, these polyphenols were present at negligible concentrations (< 2 μg/100 g sample).

Conversely, NEPA was the main source of polyphenol in samples enriched with CT which, in addition, was affected by the type of protein (P = 0.020). The overall mean content of NEPA (all times considered) in GE-SC-C (315.4 mg/100 g) was lower than that of GE-WPI-C (412.9 mg/100 g) whereas the overall mean of GE-ISP-C was not different from the other two systems (387.3 mg/100 g). The same amount of CT was added to the three systems, but it should be considered that, in order to determine NEPA content, these compounds must be released from the food matrix through a hydrolysis procedure. Indeed, it is widely known that CT develop many interactions with proteins, mostly based on hydrogen bonds and different non-covalent hydrophobic interactions (Jakobek, 2015; Le Bourvellec & Renard, 2012). Thus, depending on the kind of interactions established with the proteins and their strength, NEPA will be more or less retained within the GE, affecting their release. The interactions between CT and proteins are known to be dependent on several factors; in particular, those with high molecular weight, rich in proline and other apolar amino acids, as well as those with an open random structure, have been found to be more prone to interact with CT (Jakobek, 2015; Le Bourvellec & Renard, 2012). Regarding the proteins used in this study, SC is characteristically rich in proline and it has an open random conformation, contrary to WPI and SPI. Moreover, the molecular weight of ISP (180-350 KDa) is higher than that of caseins and WPI (19-25 KDa and 18-66 KDa, respectively) (Gómez-Estaca, Gavara, Catalá, & Hernández-Muñoz, 2016; Hu, McClements, & Decker, 2003). Accordingly, GE-WPI-Ex, made with a protein with low molecular weight than ISP and higher arginine content than SC, exhibited the highest NEPA value.

The interaction of phenolics with proteins can affect the properties of different food matrices and delivery systems containing proteins. In this connection, Freire et al (submitted) reported that the presence of hydroxytyrosol affected various rheological, textural and stability characteristics of similar cold-set GE. The authors reported that the presence of this phenolic compound lowered gravitational and cooking losses and increased hardness of those GE with hydroxytyrosol when compared with their analogues without it. In the present study, all GE showed excellent binding properties at the initial time with the exception of GE-WPI-C (4.89%) whereas in the presence of tannins (GE-WPI-Ex) there were no losses. Despite that MANOVA showed that, the

overall addition of CT led to GE with increased syneresis (P = 0.047). The protein used to form the different emulsions also affected the binding capacity of the different GE (P = 0.028) which stability can be ranked as follows: ISP>WPI>SC. Indeed, ISP is widely used in the meat industry due to its gelling properties and excellent water holding capacity whereas caseinates are known to have limited effect on WHC and do not gel (Petracci, Bianchi, Mudalal, & Cavani, 2013). In addition, whey proteins in combination with carrageenans showed different synergistic behavior on texture properties (Barbut, 2010).

Regarding antioxidant activity of the samples, this was only measured in NEPA extracts because of the low EPA content. This parameter was not affected by the employment of different proteins to form the studied delivery systems (Table 4). However, the addition of CT into the different GE improved their antioxidant capacity (P < 0.001). This fact can be attributed to the phenolic nature of CT. These well-known antioxidant properties explained the observed decreased content in lipid hydroperoxides in those systems in which CT were added (Table 4). Interestingly, the CT used in the present study presented a high degree of polymerization, being less studied than low molecular weight CT. Likewise, an increased antioxidant activity was recorded in meat systems containing high molecular weight CT (Bastida et al., 2009). Furthermore, the addition of CT also resulted in lower TBARS values (P = 0.001). Therefore, the addition of proanthocyanidins contributed to a delayed onset of oxidation in these systems due to its antioxidant properties and represents a suitable strategy to protect from oxidation these systems containing unsaturated lipids. The potential health effects for the consumer of these GE remain to be elucidated; nevertheless, the doses used in this study are nutritionally relevant.

The stability of emulsions systems is determined by several factors such as the type of emulsifier, droplet size, pH and preparation conditions (McClements & Decker, 2000). In this connection, the use of different proteins to form the emulsion systems was found to influence on lipid hydroperoxide content and TBARS values (Table 4). In general, those GE with ISP recorded higher content in lipid hydroperoxides than those elaborated with SC or WPI. With respect to TBARS, higher oxidation values were, in general, recorded in those GE with WPI when compared with those containing ISP or SC which in turn were

similar between them. Hu, McClements and Decker (2003) reported that the oxidative stability of different emulsions at pH 3 stabilized with the same proteins was in the order of CS > WPI > SPI. The overall antioxidant activity of casein-stabilized emulsions can be mainly attributed to its ability to scavenge free radicals and to physically bind transition metals (Matalanis et al., 2012).

3.2. Effect of storage time

Color changes may occur during the course of the storage and, in consequence, be detrimental with regards of the utilization of these ingredients in food products. Lightness has been shown to decrease with storage time in all systems with the exception of GE-ISP-C which remained unchanged (Table 2). The decrease of lightness normally occurred in simple emulsions, double emulsions and gelled double emulsion during their storage or as a consequence of other processing factors (Bou, Cofrades, & Jimenez-Colmenero, 2014a; Cofrades et al., 2016; Flaiz et al., 2016). Redness values were increased in GE-SC-C, GE-WPI-C, GE-WPI-Ex and GE-ISP-C with longer storage periods whereas the opposite trend was observed in GE-SC-Ex and GE-ISP-Ex. Flaiz et al. (2016) also reported an increased redness with longer storage periods in GE containing hydroxytyrosol. The authors associated the increase of redness to the release of hydroxytyrosol which was encapsulated in the inner aqueous phase. The results observed in this study can be in part attributed to the release of CT but also to their interactions with proteins. Table 2 also shows that yellowness values decreased with longer storage periods in GE-SC-Ex, GE-WPI-C, GE-WPI-Ex and GE-ISP-Ex. However, yellowness tended to increase in GE-ISP-C with longer storage periods and GE-SC-C followed an unclear trend as *b** values progressively decrease until 21 days of storage and after 28 days increased till reaching similar values to those recorded at the beginning of the storage.

Overall, these color changes with storage can be considered of minor relevance when compared with those caused by the presence of CT or the nature of the protein used to stabilize the different emulsions. Given that these GE can be considered as relatively stable under the studied storage conditions their potential use as fat replacers seems to

be guaranteed as they are expected to be used for the intended food application immediately or stored for as short a time as possible for a number of reasons (economics, organization of production tasks, safety, etc.).

In general, the pH of all GE systems decreased upon storage (Table 3). This decrease was more pronounced in those systems without CT. The addition of this ingredient resulted in minimal or no changes during storage. These results are in line with those observed in similar GE containing encapsulated hydroxytyrosol (Freire submitted). Regarding EPA and NEPA content in the systems, there was, in general, a small increase in EPA content with longer storage periods which can be attributed to a higher accessibility of some NEPA during storage, being transferred to the EPA fraction (Table 3). Despite that, EPA values remained negligible during the whole study period. In the case of NEPA, no clear tendency was observed during storage. It should be considered that CT-proteins interactions do not follow the classic "lock and key" model, but a more complex one (Watrelot, Renard, & Le Bourvellec, 2015). Therefore, during the storage period, NEPA are affected by several processes (Kroll, Rawel, & Rohn, 2003; Le Bourvellec & Renard, 2012). These may involve the decrease NEPA content as is the case of oxidation, which generates quinones and other forms that establish irreversible interactions with proteins, and the apparition of stronger associations with proteins and auto-association between CT. On the contrary, the weakening of the bonds in other sections of the GE may cause increased NEPA levels. Finally, it should not be disregarded that the methodology for NEPA determination (Pérez-Jiménez et al., 2009) was developed for foods of vegetable origin, with low fat content. Although the overall results obtained here (lack of detection in samples without CT and detection at the same range in all samples containing it) show its validity for other kind of food systems, specific adaptations might improve its applicability for future studies.

In relation to these systems binding capacity, it can be observed that losses were increased with longer storage periods in those GE containing SC (Table 3). Despite that, the comparison between GE-SC-C and GE-SC-Ex indicates that the addition of CT caused higher losses. Conversely, GE-WPI-C recorded higher losses at the initial time but with storage the emulsion stability increased and no losses were observed at the end of the storage period. The addition of tannins in GE-WPI-Ex overcame the observed initial

losses in the latter system. With regards to those systems elaborated with ISP, it can be observed that the presence of CT in GE-ISP-Ex resulted in increased losses with longer storage periods whereas GE-ISP-C recorded minimal losses at the initial time. Nevertheless, all systems presented appropriate binding properties and thus may be considered suitable as food ingredients and fat replacers (Cofrades, Ayo, Serrano, Carballo, & Jimenez-Colmenero, 2006).

In general, these results are difficult to interpret but the observed effects can be attributed to the different formulations and thus to the proteins used to stabilize these GE, the presence of CT and the interactions between the different food components throughout storage, which, as described above, take place in different directions. Upon storage, the antioxidant activity of the systems was maintained or reduced in all GE with the exception of GE-SC-C which recorded lower values at the initial time. The progression of oxidation entails a reduction of the system's ability to scavenge radicals. Therefore, it appears that these systems are relatively stable throughout the storage time. In this connection, the content in lipid hydroperoxides remained unchanged or was even reduced in those systems containing CT (Table 4). Conversely, GE-WPI-C and GE-ISP-C recorded slightly higher values at the end of the storage. The GE-SC-C recorded higher content in lipid hydroperoxides at 14 days of storage and thereafter decreased until reaching similar values to those observed at the initial time. In general, the content in primary oxidation compounds can be regarded as low.

At the beginning of the storage, the addition of CT reduced TBARS values in GE-SC-Ex with respect to its homologue (GE-SC-C). However, the oxidation values remained unchanged over the course of the storage whereas in the case of GE-SC-Ex they progressively increased till reaching the same values at the end the storage. In general, those systems containing WPI recorded similar oxidation patterns reaching higher TBARS values at the end of the storage. Those GE containing ISP recorded a similar behavior in which the initial TBARS values were increased from 7 to 21 days of storage and thereafter decreased after 28 days. Overall, the addition of CT resulted in lower TBARS values at the end of the storage when comparing with their respective systems. As previously discussed, it is possible that the interaction and even polymerization between CT and proteins was more pronounced in GE-SC-Ex and GE-ISP-Ex than in GE-

WPI-Ex. The oxidation in emulsions systems mainly occurs at the oil/water interface (McClements & Decker, 2000). Therefore, in the case of GE-WPI-Ex it is possible that the limited interaction of CT implied their localization far from the lipid phase and thus contributes to explain the observed higher TBARS values in this system.

The different location of CT and their changes during storage may also help to explain certain differences in the emulsion stability of the studied GE. Lipids may be protected from oxidation in the presence of CT which might be oxidized to quinones and polymerize with proteins and, in turn, affect to the stability of the emulsion. In this regard, Freire et al (submitted) reported that the presence of hydroxytyrosol in a GE similar to that containing SC led to a less rigid protein gel structure but caused lower losses at the end of the storage when compared with a control without this phenolic compound. Despite that, the recorded oxidation values in these GE systems can be regarded as low, and even more considering their content in unsaturated fatty acids which are well-known to be prone to oxidize, and therefore guarantee their use as fat replacers.

4. CONCLUSIONS

The design of GE represents an interesting strategy for the development of foods with healthier lipid composition (reduced fat content and rich in n-3 fatty acids). The overall appearance of GE, binding properties and oxidative stability are suitable for their use as fat replacers regardless of the protein used to stabilize the emulsions. Moreover, the design of these delivery systems allows the incorporation of other bioactive compounds such as CT. Even though its addition causes color changes in GE, these systems are still suitable as functional ingredients and, in particular, as fat replacers. Moreover, the addition of CT may help to prevent oxidation in those systems that are particularly prone to oxidize. In this regard, the antioxidant capacity of CT and its interaction with the other components of these GE may explain the observed effects on GE binding properties and their susceptibility towards oxidation.

Acknowledgements:

The authors wish to thank the Spanish Ministry of Economy and Competitiveness for Ms. María Freire's predoctoral fellowship BES-2012-057977. Dr. R. Bou has been supported by a contract from the JAE-postdoctoral (CSIC) Program financed in part through the European Social Fund (ESF).

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Table 1. Composition of the aqueous and lipid phases used in the elaboration of the different oil-in-water (O/W) emulsions and final ingredients used to obtain the different gelled emulsions.

GE-SC-C	GE-SC-Ex	GE-WPI-C	GE-WPI-Ex	GE-ISP-C	GE-ISP-Ex
Composition of the aqueous phase (100 g)					
2 g sodium caseinate	2 g sodium caseinate	6 g whey protein isolate	6 g whey protein isolate	3 g isolated soy protein	3 g isolated soy protein
0.02 g sodium azide	0.02 g sodium azide	0.02 g sodium azide	0.02 g sodium azide	0.02 g sodium azide	0.02 g sodium azide
Composition of the lipid phase (100 g)					
44.39 g olive oil	44.39 g olive oil	44.39 g olive oil	44.39 g olive oil	44.39 g olive oil	44.39 g olive oil
37.87 g linseed oil	37.87 g linseed oil	37.87 g linseed oil	37.87 g linseed oil	37.87 g linseed oil	37.87 g linseed oil
17.74 g fish oil	17.74 g fish oil	17.74 g fish oil	17.74 g fish oil	17.74 g fish oil	17.74 g fish oil
Addition of condensed tannins and remaining ingredients to gel 100 g of O/W emulsion					
	3.0 g Exxenterol®		3.9 g Exxenterol®		3.9 g Exxenterol®
30 g deionized water	30 g deionized water	30 g deionized water	30 g deionized water	30 g deionized water	30 g deionized water
0.5 g bovine gelatin	0.5 g bovine gelatin	0.5 g bovine gelatin	0.5 g bovine gelatin	0.5 g bovine gelatin	0.5 g bovine gelatin
0.3 g k-carrageenan	0.3 g k-carrageenan	0.3 g k-carrageenan	0.3 g k-carrageenan	0.3 g k-carrageenan	0.3 g k-carrageenan
1.5 g transglutaminase	1.5 g transglutaminase	1.5 g transglutaminase	1.5 g transglutaminase	1.5 g transglutaminase	1.5 g transglutaminase

The oil-in-water emulsions (O/W) were prepared by mixing the same amount of aqueous and lipid phases (50g/100g). Condensed tannins were after the elaboration of the emulsions and immediately gelled by adding the reported ingredients in 100 g of emulsion. The abbreviations GE-SC-C, GE-SC-Ex, GE-WPI-C, GE-WPI-Ex, GE-ISP-C and GE-ISP-Ex correspond to the gelled emulsions (GE-) prepared with sodium caseinate (SC-), whey protein isolate (WPI-) and isolated soy protein (ISP-) as emulsifiers with and without the presence of condensed tannins (Ex).

Table 2. Instrumental color parameters for lightness (L*), redness (a*) and yellowness (b*) of the gelled emulsions.

		GE-SC-C	GE-SC-Ex	GE-WPI-C	GE-WPI-Ex	GE-ISP-C	GE-ISP-Ex	Effect of the emulsifier	Effect of tannins	Interaction
L*	1	87.00±0.05^{b,6}	68.99±0.15^{e,3}	84.96±0.15^{d,5}	65.26±0.24^{d,2}	80.67±0.33^{a,4}	48.94±0.88^{d,1}	**	**	**
	7	87.11±0.04^{b,6}	67.56±0.19^{d,3}	85.07±0.14^{d,5}	64.05±0.21^{c,2}	80.69±0.41^{a,4}	47.83±0.27^{c,1}			
	14	85.83±0.32^{a,6}	65.96±0.24^{c,3}	84.64±0.14^{c,5}	63.48±0.73$^{c-d,2}$	80.07±0.17^{a,4}	46.99±0.26^{b,1}			
	21	85.80±0.46^{a,6}	65.34±0.09^{b,3}	84.56±0.08$^{a-b,5}$	62.75±0.44$^{a-b,2}$	80.37±0.60^{a,4}	46.62±0.12$^{a-b,1}$			
	28	85.59±0.30^{a,6}	64.08±0.13^{a,3}	84.35±0.16^{a,5}	62.66±0.50^{a,2}	79.94±0.62^{a,4}	46.23±0.24^{a,1}			
a*	1	-0.45±0.01$^{a,1-2}$	2.71±0.04^{e,3}	-0.51±0.01^{a,1}	3.09±0.06^{a,4}	-0.36±0.02^{a,2}	5.24±0.12$^{b-c,5}$	**	**	**
	7	-0.42±0.02$^{b,1-2}$	2.54± 0.06^{d,3}	-0.48±0.01^{b,1}	3.25±0.07^{b,4}	-0.24±0.01^{b,2}	5.27±0.10^{c,5}			
	14	-0.43±0.00^{b,1}	2.38±0.06^{c,3}	-0.36±0.01$^{c,1-2}$	3.35±0.07$^{b-c,4}$	-0.24±0.01^{b,2}	5.24±0.16$^{b-c,5}$			
	21	-0.27± 0.00^{d,2}	2.26±2.26^{b,4}	-0.45±0.01^{d,1}	3.37±0.06^{c,5}	-0.13±0.01^{c,3}	5.10±0.06^{b,6}			
	28	-0.29±0.01^{c,2}	1.93±0.03^{a,3}	-0.39±0.01^{e,1}	3.40±0.06^{c,4}	-0.24±0.01^{b,2}	4.86±0.05^{a,5}			
b*	1	12.19±0.02^{c,4}	6.51±0.09^{e,1}	12.83±0.05$^{c-d,5}$	6.80±0.12^{c,2}	19.28±0.07$^{a-b,6}$	10.77±0.32^{d,3}	**	**	**
	7	11.98±0.04^{b,4}	5.63±0.04^{d,1}	12.95±0.10^{d,5}	6.67±0.07$^{b-c,2}$	19.13±0.14^{a,6}	10.36±0.14^{c,3}			
	14	11.98±0.05^{b,4}	5.50± 0.09^{c,1}	12.61± 0.10$^{a-b,5}$	6.67± 0.05$^{b-c,2}$	19.30± 0.06$^{a-b,6}$	9.99± 0.20^{b,3}			
	21	11.77±0.05^{a,4}	5.27±0.05^{b,1}	12.75±0.11$^{b-c,5}$	6.52±0.10^{a,2}	19.35±0.05^{b,6}	9.58±0.03^{a,3}			
	28	12.12±0.07^{c,4}	5.08±0.11^{a,1}	12.53±0.09^{a,5}	6.54±0.10$^{a-b,2}$	19.44±0.15^{b,6}	9.35±0.16^{a,3}			

See Table 1 for treatment abbreviations. Results are means ± standard deviation (n = 180) of the studied gelled emulsions. Different numbers in the same row or letters in the same column indicate significant differences according to series of one-way ANOVA (P < 0.05). A MANOVA was conducted to study whether the factors “type of emulsifier” and “addition of condensed tannins”, and the interaction between them caused significant effects among the different gelled emulsions. *P<0.05, **P<0.01.

Table 3. Emulsion gels pH, extractable proanthocyanidins (EPA), non-extractable proanthocyanidins (NEPA) and syneresis.

		GE-SC-C	GE-SC-Ex	GE-WPI-C	GE-WPI-Ex	GE-ISP-C	GE-ISP-Ex	Effect of the emulsifier	Effect of tannins	Interaction
pH	1	8.43±0.03^{c,5}	7.11±0.01^{b,2}	7.62±0.03^{d,4}	7.00±0.01$^{b-c,1}$	8.98±0.03$^{c-d,6}$	7.30±0.01^{a,3}	**	**	**
	7	8.19±0.01^{a,5}	7.10±0.02$^{a-b,2}$	7.53±0.02^{c,4}	7.04±0.01^{c,1}	9.04±0.01^{d,6}	7.36±0.01^{c,3}			
	14	8.27±0.03^{b,4}	7.14±0.02$^{b-c,2}$	7.34±0.02^{b,3}	6.96±0.04$^{a-b,1}$	8.89±0.03^{c,5}	7.34±0.02$^{b-c,3}$			
	21	8.27±0.02^{b,4}	7.16±0.01^{c,2}	7.30±0.01$^{a-b,3}$	6.91±0.03^{a,1}	8.76±0.03^{b,5}	7.31±0.01^{a,3}			
	28	8.22±0.01$^{a-b,3}$	7.06±0.02^{a,1}	7.27±0.02^{a,2}	6.97±0.01$^{a-b,1}$	8.46±0.10^{a,4}	7.31±0.01$^{a-b,2}$			
EPA (µg gallic acid/ 100g sample)	1	17±2^{a,1}	156±1^{b,4}	9±1^{a,1}	82±11^{a,3}	10±3^{a,1}	54±9^{a,2}		**	
	7	17±12^{a,1}	122±31$^{a-b,2}$	13±1^{a,1}	119±14^{b,2}	11±2^{a,1}	105±4^{b,2}			
	14	64±4^{b,1}	87±9$^{a,2-3}$	71±6^{b,2}	138±13^{b,4}	44±2^{b,1}	104±12^{b,3}			
	21	49±11$^{a-b,1}$	100±2^{a,2}	60±7^{b,1}	146±5^{b,3}	43±1^{b,1}	98±11$^{b,2-3}$			
	28	69±34^{b,1}	155±18^{b,3}	61±3^{b,1}	151±19^{b,3}	89±5$^{c,1-2}$	124±10$^{b,2-3}$			
NEPA (mg NEPA/ 100g sample)	1	0.00±0.00^{a,1}	423±1^{e,3}	0.00±0.00^{a,1}	408±10^{c,2}	0.00±0.00^{a,1}	523±13^{d,4}	*	**	*
	7	0.00±0.00^{a,1}	305±6^{c,4}	0.00±0.00^{a,1}	271±3^{a,3}	0.00±0.00^{a,1}	261±5^{a,2}			
	14	0.00±0.00^{a,1}	236±3^{a,2}	0.00±0.00^{a,1}	322±4^{b,3}	0.00±0.00^{a,1}	369±16^{b,4}			
	21	0.00±0.00^{a,1}	321±4^{d,2}	0.00±0.00^{a,1}	448±10^{d,4}	0.00±0.00^{a,1}	315±5^{b,3}			
	28	0.00±0.00^{a,1}	293±3^{b,2}	0.00±0.00^{a,1}	615±18^{e,4}	0.00±0.00^{a,1}	441±26^{c,3}			
Syneresis (%)	1	0.00±0.00^{a,1}	0.00±0.00^{a,1}	4.89±0.86^{b,2}	0.00±0.00^{a,1}	0.20±0.00^{b,1}	0.00±0.00^{a,1}	*	*	**
	7	0.00±0.00^{a,1}	2.31±0.36^{b,2}	1.02±1.77$^{a,1-2}$	0.00±0.00^{a,1}	0.00±0.00^{a,1}	0.17±0.04$^{a-b,1}$			

14	0.07±0.07^{a,1}	2.06±1.04$^{a-b,2}$	0.00±0.00^{a,1}	0.00±0.00^{a,1}	0.00±0.00^{a,1}	0.65±0.09$^{b-c,1}$
21	0.00±0.00^{a,1}	2.32±1.08^{b,2}	0.13±0.23^{a,1}	0.00±0.00^{a,1}	0.00±0.00^{a,1}	0.89±0.52$^{c-d,1}$
28	0.32±0.03$^{b,1-2}$	3.55±0.88^{b,3}	0.00±0.00^{a,1}	0.04±0.06^{a,1}	0.00±0.00^{a,1}	1.16±0.04^{d,2}

See Table 1 for treatment abbreviations. Results are means ± standard deviation (n = 120) of the studied gelled emulsions. Different numbers in the same row or letters in the same column indicate significant differences according to series of one-way ANOVA ($P < 0.05$). A MANOVA was conducted to study whether the factors “type of emulsifier” and “addition of condensed tannins”, and the interaction between them caused significant effects among the different gelled emulsions. *P<0.05, **P<0.01.

Table 4. Emulsion gels antioxidant capacity, content in lipid hydroperoxides (PV) and TBARS values.

		GE-SC-C	GE-SC-Ex	GE-WPI-C	GE-WPI-Ex	GE-ISP-C	GE-ISP-Ex	Effect of the emulsifier	Effect of tannins	Interaction
Antioxidant capacity (µg Trolox eq./mg sample)	1	0.10±0.02^{a,1}	3.92±0.43^{a,3}	0.44±0.10^{b,1}	1.60±0.03$^{a,1-2}$	0.46±0.05^{a,1}	7.22±0.12$^{b,2-3}$		**	*
	7	0.24±0.01^{b,1}	4.49±1.44$^{a,1-2-3}$	0.18±0.03$^{a,1-2}$	1.90±0.77^{a,3}	0.81±0.01^{b,1}	2.93±0.28$^{a,2-3}$			
	14	0.23±0.07^{b,1}	1.89±0.95$^{a,2-3}$	0.47±0.01^{b,1}	3.34±0.06^{b,2}	0.44±0.20^{a,1}	2.35±1.18^{a,3}			
	21	0.28±0.03^{b,1}	2.71±0.96$^{a,2-3}$	0.49±0.05^{b,1}	1.89±0.12^{a,2}	0.31±0.09^{a,1}	3.40±0.12^{a,3}			
	28	0.10±0.02^{a,1}	3.92±0.43^{a,3}	0.44±0.10^{b,1}	1.60±0.03$^{a,1-2}$	0.46±0.05^{a,1}	7.22±0.12$^{b,2-3}$		**	*
PV (mmol hydroperoxyde/kg oil)	1	1.04±0.05$^{a-b,3}$	0.94±0.06$^{b,2-3}$	0.93±0.01$^{a-b,2}$	0.89±0.04$^{c,1-2}$	1.04±0.01^{a,3}	0.80±0.01^{a,1}	**	**	
	7	0.80±0.04^{a,1}	0.67±0.05^{a,1}	0.73±0.25^{a,1}	0.80±0.09$^{b-c,1}$	1.01±0.04^{a,1}	0.85±0.15^{a,1}			
	14	1.22±0.25^{b,1}	0.98±0.15$^{b,1-2}$	0.74±0.20^{a,1}	0.60±0.02$^{a-b,1}$	1.21±0.01$^{a-b,2}$	0.88±0.13$^{a,1-2}$			
	21	0.72±0.02^{a,1}	0.58±0.03^{a,1}	0.66±0.09^{a,1}	0.59±0.12^{a,1}	1.50±0.42$^{a-b,2}$	0.93±0.07^{a,1}			
	28	0.85±0.08^{a,2}	0.57±0.02^{a,1}	1.21±0.02^{b,3}	0.55±0.07^{a,1}	1.59±0.06^{b,4}	0.86±0.05^{a,2}			
TBARS (mg MDA/kg oil)	1	4.47±1.36^{a,3}	2.34±0.13^{a,1}	2.83±0.24$^{a,1-2}$	4.16±0.02$^{b,2-3}$	4.12±0.15$^{a,2-3}$	2.50±0.24^{a,1}	**	**	
	7	4.04±0.98^{a,1}	3.63±0.82^{b,1}	2.73±0.06^{a,1}	2.64±0.12^{a,1}	6.34±0.09^{b,2}	3.34±0.26^{b,1}			
	14	4.53±0.42$^{a,1-2}$	3.86±0.32^{b,1}	5.18±0.09^{b,2}	5.17±0.08^{b,2}	8.65±0.38^{c,2}	3.81±0.06^{b,3}			
	21	4.40±0.24^{a,1}	4.58±0.28$^{b-c,1}$	12.81±0.83^{c,3}	9.16±0.76^{c,2}	9.26±0.79^{c,2}	3.35±0.57^{b,1}			
	28	5.94±0.16^{a,3}	5.22±0.33$^{c,2-3}$	13.97±0.03^{d,5}	11.50±0.48^{d,4}	5.13±0.36^{a,2}	1.82±0.13^{a,1}			

See Table 1 for treatment abbreviations. Results are means ± standard deviation (n = 120) of the studied gelled emulsions. Different numbers in the same row or letters in the same column indicate significant differences according to series of one-way ANOVA ($P < 0.05$). A MANOVA was conducted to study whether the factors "type of emulsifier" and "addition of condensed tannins", and the interaction between them caused significant effects among the different gelled emulsions. *P<0.05, **P<0.01.

Figure 1. Comparison of gelled double emulsions stabilized with whey protein isolate and in the absence (left) and presence of condensed tannins (right).