Modified phosphatidylcholine with different alkyl chain length and covalently attached caffeic acid affects the physical and oxidative stability of omega-3 delivery 70% oil-in-water emulsions

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Highlights

- Increased amount of modified phosphatidylcholine(PC)s improved physical stability
- Modified PCs enhanced antioxidant activity at the oil-water interface
- Having caffeic acid attached to PC significantly improved oxidative stability
- Modified PC_C16 led to a higher surface protein load compared to PC_C14

Modified phosphatidylcholine with different alkyl chain length and covalently attached caffeic acid affects the physical and oxidative stability of omega-3 delivery 70% oil-in-water emulsions

Betül Yesiltas[1], Ann-Dorit M. Sørensen[1], Pedro J. García-Moreno[1], Sampson Anankanbil[2], Zheng Guo[2], Charlotte Jacobsen[1]

[1]National Food Institute, Technical University of Denmark, Lyngby, Denmark

[2]Department of Engineering, Aarhus University, Aarhus, Denmark

*Correspondence: Charlotte Jacobsen, National Food Institute, Technical University of Denmark, Kemitorvet, Building 204, 2800 Kgs. Lyngby, Tel: +45 23 27 90 75; e-mail: chja@food.dtu.dk

Abstract

This study investigated the effects of modified phosphatidylcholine (PC) with different alkyl chain lengths (PC_C14 and PC_C16) and covalently attached caffeic acid on the physical and oxidative stability of 70% fish oil-in-water emulsions. High fat emulsions were produced using different amounts of modified PCs in combination with sodium caseinate and soy-PC. Results showed that the physical stability of the emulsions was improved with increasing concentrations of modified PCs, due to their high surface activity. Emulsion stabilized with PC_C14 led to smaller droplets and higher viscosity, whereas PC_C16 had higher protein surface load, which may result in a thicker interfacial layer. Modified PCs enhanced the oxidative stability of the emulsions due to the attachment of caffeic acid to the glycerol backbone of PC, which brings the antioxidant in the vicinity of oil-water interface. PC_C16 led to less formation of primary and secondary oxidation products compared to PC_14 at their equivalent concentrations.

Key words: modified phospholipids; phosphatidylcholine; sodium caseinate; lipid oxidation; oil-water interface; caffeic acid; high fat delivery emulsions

1. Introduction

Evidences for health benefits of long chain (LC) omega-3 (n-3) polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) have been increasing in the last decades. Some of the effects include decreasing the risk of cardiovascular diseases, improving brain development in infants, maintaining normal blood pressure and triglyceride levels in the blood, supporting mental health as well as immune system (Calder & Yaqoob, 2012; Song et al., 2016; Cheatham, Colombo, & Carlson, 2006).

LC n-3 PUFAs are mainly available in marine fish and fish products. However, the consumption of these LC n-3 PUFAs is inadequate in most Western countries (EFSA, 2010). Therefore, increasing the consumption of these bioactive compounds has attracted interest from food researchers and industry. Due to easier hydrogen abstraction from the bis-allylic positions, LC n-3 PUFAs are highly prone to oxidation (Frankel, 2012b). Oxidation causes loss of nutritional value and the formation of undesired off-flavors. Therefore, delivery systems have been developed in order to protect LC n-3 PUFAs against lipid oxidation. One of the delivery systems for LC n-3 PUFAs is oil-in-water emulsions. High fat (70%) oil-in-water emulsions were found to be advantageous for enrichment of high fat content food products with LC n-3 PUFAs (Horn et al., 2011; Yesiltas et al., 2018a,b).

Previous studies focused on different ways of improving physical properties of the oil-water interface as well as enhancing the oxidative stability of the oil-in-water emulsion system. The strategies studied include to: i) use phenolipids with various alkyl chain lengths and phenolic compounds (Laguerre et al., 2009; Sørensen et al., 2014; Alemán et al., 2015; Sørensen et

al., 2017), ii) incorporate emulsifiers with antioxidant activities (Yesiltas et al., 2019), iii) add free antioxidants in the emulsion along with emulsifiers (Sørensen et al., 2008), or iv) to have emulsifiers modified with various alkyl chains and covalently attached phenolic acids (Anankanbil et al., 2018; Yesiltas et al., 2018b).

One of the approaches to limit lipid oxidation whilst ensuring an acceptable physical stability is engineering an interfacial layer with optimal properties to resist oxidation, as oxidation has been claimed to be initiated at the interface (Berton-Carabin et al., 2014). In order to have a bioinspired interface structure, proteins and phospholipids could be used in combination. Such a combination of emulsifiers has been suggested to form an interface structure providing a better coverage of the oil droplets compared to a single emulsifier (Fang and Dalgleish, 2016; García-Moreno et al., 2014; Berton-Carabin et al., 2018; Yesiltas et al., 2019), which could provide a good physical barrier for prooxidants` diffusion into the oil phase.

Previous studies have focused on the antioxidant activity at the oil-water interface of the oil-in-water emulsions (Laguerre et al., 2013; Berton-Carabin et al., 2014, 2018). However, lipophilicity of the surface active antioxidative compound has an important effect on its antioxidative activity in heterogeneous systems. Based on polar paradox and cut-off effect theories, researchers have focused on finding the most efficient lipophilicity for particular surface active compounds with antioxidant activity in emulsion systems. In the studies conducted using phenolipids with various lipophilicity, it was observed that the most efficient alkyl chain lengths could be system and particular compound dependent (Laguerre et al., 2013; Alemán et al., 2015; Sørensen et al., 2017).

Caffeic acid is a commonly used antioxidant with radical scavenging and metal chelating (especially iron) activities and its efficacy has been shown to be dependent on pH, addition of iron and emulsifier type (Gülcin, 2006; Sørensen et al., 2008). The antioxidative properties (radical scavenging and reducing power) of caffeic, ferulic and coumaric acids and their derivatives were reported in a previous study. Caffeic acid and caffeates had the highest radical scavenging and metal chelating activities, whereas the lowest radical scavenging and metal chelating activities were measured for coumaric acid and coumarates (Sørensen et al., 2014). The effect of the alkyl chain length on antioxidant efficacy has also been studied for caffeic acid esters. It was found that the medium alkyl chain length caffeic acid (octyl caffeate) had higher antioxidant activity compared to shorter and longer alkyl chain lengths in 40% soybean oil-in-water emulsions (Costa, Losada-Barreiro, Paica-Martinsa, and Bravo-Díaz, 2017). Similar results were obtained when alkyl caffeates were applied in fish oil enriched mayonnaise; short to medium alkyl chain (C4, C8, and C12) caffeic acid were found to be more effective antioxidants (Alemán et al., 2015). Another study carried out with modified diacetyl tartaric acid esters of mono- and diglycerides (DATEM) with covalently attached caffeic acid and C12 or C14 alkyl chain lengths showed that the modified DATEM with C14 chain length provided slightly better oxidative stability compared to the modified DATEM with C12 chain length in 70% fish oil-in-water emulsions produced with sodium caseinate (CAS), DATEM and modified DATEMs (Yesiltas et al., 2018b).

The aim of this study was to investigate the effect of modified phosphatidylcholine (PC) with covalently attached caffeic acid and different alkyl chain lengths on physical and oxidative stability of 70% fish oil-in-water emulsions stabilized by sodium caseinate, soy PC and

105 modified PCs. Soy PC and modified PC in combination with CAS was used in order to
106 decrease the viscosity of the high fat omega-3 delivery emulsions when compared to
107 emulsions stabilized only with CAS. This will make it easier to apply the delivery emulsions in
108 food systems. It was hypothesized that incorporation of modified PCs in the 70% fish oil-in-
109 water emulsions will lead to a more pronounced reduction in interfacial tension compared to
110 conventional PC with 2 alkyl chains. Thus, this will result in emulsions with enhanced physical
111 stability. Moreover, different chain length (C14 and C16) of modified PCs is expected to have
112 an impact on adsorption of the molecule at the oil-water interface of the emulsions due to the
113 different hydrophilic-lipophilic balance (HLB) values. In addition, modified PCs with covalently
114 attached caffeic acid are expected to enhance oxidative stability of the emulsions compared
115 to physical mixtures of soy PC and free caffeic acids with the strategy of bringing the phenolic
116 compounds with antioxidant activity into the vicinity of the interface taking advantage of the
117 amphiphilicity of the PCs.

118 ## 2. Materials and Methods

119 ### 2.1. Materials

120 Cod liver oil was donated by Vesteraalens A/S (Sortland, Norway) and kept at -40ºC until use.
121 Peroxide value of the cod liver oil was 0.09 ± 0.00 meq peroxides/kg oil. The fatty acid
122 content of the fish oil (%, w/w) was as follows: C14:0 (4.0), C16:0 (9.2), C16:1 n-7 (8.3),
123 C18:0 (2.2), C18:1 n-9 (15.8), C18:1 n-7 (4.1), C18:2 n-6 (2.5), C18:3 n-3 (0.2), C20:1 n-9
124 (11.4), C20:5 n-3 (8.8), C22:1 n-11 (5.4), and C22:6 n-3 (11.4). Alpha-, beta-, gamma-, and
125 delta tocopherol contents were 146±7, 0±0, 97±2, 43±0.3 μg toc/g oil, respectively. Sodium

126 caseinate (Miprodan 30) was provided by Arla Foods Ingredients amba (Viby J, Denmark).
127 Protein content of the sodium caseinate was reported as 92% by Arla.

128 Phosphatidylcholine extracted from soybean (LIPOID S100, soy PC) was donated by Lipoid
129 GmbH, Germany. Peroxide value of phosphatidylcholine was 1.91 meq peroxides/kg sample.
130 It was reported in the analysis of certificate of the LIPOID S100 that 97.1% of the product was
131 phosphatidylcholine (based on dry weight) and contained 0.20% DL-α-Tocopherol, which is
132 an antioxidant. Other lipid molecules were also reported as follows: 1.2%
133 lysophosphatidylcholine, 0.5% N-acyl-phosphatidylethanolamine, ≤0.1%
134 phosphatidylethanolamine, ≤0.1% phosphatidylinositol, 0.8% non-polar lipids, and 0.3%
135 triglycerides. The fatty acid (%, w/w) content of the soy PC (LIPOID S100) was analyzed and
136 found as follows: C14:0 (0.09), C16:0 (12.55), C18:0 (3.77), C18:1 n-9 (8.42), C18:2 n-6
137 (65.82), C18:3 n-6 (7.11). Caffeic acid was purchased from Sigma Aldrich. Modified PCs
138 (PC_C14 and PC_C16) with different alkyl chains C14 or C16 and caffeic acid were
139 synthesized as described in a previous study (Anankanbil et al., 2018). The fatty acid content
140 (%, w/w) of PC_C14 and PC_C16 were C14:0 (99.38%) and C16:0 (98.74%), respectively. All
141 solvents and chemicals used were of analytical grade.

142 **2.2. Experimental design**

143 Table 1 shows the sample codes, descriptions and amounts of ingredients. All emulsion
144 samples include 70% w/w fish oil and 2.8% w/w total emulsifier with a 1.2 CAS: total PC ratio.
145 These values were determined based on a previous study (Yesiltas et al., 2019). Soy PC was
146 replaced by modified PCs in order to obtain various final caffeic acid concentrations, namely

360, 1080 and 2160 ppm in the final emulsion. Emulsions produced with soy PC and free caffeic acid were included as controls.

2.3. Emulsion preparation, storage and sampling

Aqueous phases were prepared by dissolving emulsifiers (CAS, PC, and modified PCs) in distilled water and stirred overnight at 4˚C. Before emulsification, aqueous phases were adjusted to pH 7 using 2M NaOH. Emulsions were produced in 500 g batches using a Stephan Universal mixer (Stephan, UMC5, 1995, Hameln, Germany) as described by Horn et al. (2011). Ferrous (Fe^{2+}, 100 µM) and sodium azide (0.05% w/v) were added into emulsions in order to accelerate lipid oxidation and prevent microbial growth, respectively. All emulsions were divided into 100 mL bottles in approximately 90 g and stored for 12 days at room temperature in darkness. Physical and oxidative stability parameters were assessed at days 0, 2, 5, 8 and 12.

2.4. Methods for characterization of emulsions

2.4.1. Creaming index

Creaming rate was determined on days 1, 5, 8 and 12 in the stored bottles without replicates by the measurement of creaming index. Creaming index (CI) was evaluated using equation 1:

$$CI\ (\%) = \frac{b}{a} \times 100 \qquad \text{(Equation 1)}$$

where (a) is the height of total emulsion and (b) is the height of aqueous phase which is separated at the bottom of the bottle. Creaming index was presented as in percentage.

2.4.2. Interfacial tension of the emulsifier combinations – pendant drop method

The dynamic interfacial tension of the emulsifiers at the oil-water interface was determined using an automated drop tensiometer OCA20 (DataPhysics Instruments GmbH, Filderstadt, Germany) at 25°C. Aqueous phases of emulsions 1 CAS, 2 PC_com, 5 PC_C14_2160 and 8 PC_C16_2160 (codes explained in Table 1) were prepared as described before and measured in duplicates. The surfactant solution (aqueous phase) was filled into a syringe with a screwed needle. For each measurement, a small drop of the aqueous phase sample solution was generated using the automated syringe into a quartz glass cuvette filled with MCT oil (WITARIX® MCT 60/40, IOI Oleo GmbH, Hamburg, Germany). The image of the drop was recorded with a camera every 10 s for 15 min. The images were transferred to the drop shape analysis software. The calculation of the interfacial tension was based on the shape analysis of a pendant drop according to the Young-Laplace equation, equation 2:

$$\Delta P = \gamma \left(\frac{1}{R_1} + \frac{1}{R_2} \right) \quad \text{(Equation 2)}$$

where ΔP (mN/m^2) is the pressure difference across the interface, γ (mN/m) is the interfacial tension and R_1 and R_2 (m) are the principal radii of curvature of the pendant drop. Measurements were carried out in duplicate. Changes in the interfacial tension (mN/m) were plotted as a function of time (min) for each aqueous phase solution.

2.4.3. Droplet size

Particle size of the emulsions was performed using laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) on days 0 and 12 according to the method described by Horn et al. (2011). Results were calculated as the surface weighted (D[3,2]) and

volume weighted (D[4,3]) mean diameter, which were calculated based on the equations 3 and 4, respectively:

$$D[3,2] = \Sigma n_i d_i^3 / \Sigma n_i d_i^2 \quad \text{(Equation 3)}$$

$$D[4,3] = \Sigma n_i d_i^4 / \Sigma n_i d_i^3 \quad \text{(Equation 4)}$$

where n is the number of droplets with a specific diameter, d is the diameter of the droplet and, i represents the size class of the droplets. Samples were measured in duplicates.

2.4.4. Relative protein content in the aqueous phase and the surface load of proteins

Protein content of the aqueous phase was determined based on the method described by Jacobsen, Meyer, & Adler-Nissen (1998). Emulsion sample (~20 g) was centrifuged for 10 min at 25,400g and 10°C (Sorvall RC-6 PLUS, Thermo Fisher Scientific, Osterode, Germany; rotor SS-34) to separate the aqueous phase and oil phase. Supernatant (oil phase) was removed by the use of a pipette. The rest was mixed with distilled water (1:1) and then subjected to ultracentrifugation (Beckman Ultracentrifuge L8-60M, Fullerton, CA; rotor 21102) for 1 h at 106,979g and 15 °C. Protein content of the aqueous phase was determined by the Dumas method (Elementar, Mt. Laurel, NJ, USA). Approximately 1 g of aqueous phase was placed in the sample tray and further steps were automated including sample combustion in a chamber at a high temperature (900°C) in the presence of oxygen. Content of crude protein was estimated by using a conversion factor (6.25). Protein content (g) was calculated in the aqueous phase. Measurements were performed in duplicate.

The surface load of proteins (Γ, mg/m^2) was calculated according to the equation described by Zhu et al. (2018), equation 5:

$$\Gamma = \frac{Vc(C_{INI}-C_{SER})}{S\,V_{OIL}} = \frac{(1-\Phi)d_{32}}{6\Phi}\,(C_{INI}-C_{SER}) \qquad \text{(Equation 5)}$$

where V_C and V_{OIL} are the volume of the aqueous and oil phase (mL), S is the surface area of the emulsion droplets (m^2), Φ is the oil phase volume fraction, C_{INI} is the initial concentration of the protein in the aqueous phase (mg/L), and C_{SER} is the non-adsorbed protein concentration in the aqueous phase after emulsification (mg/L).

2.4.5. Zeta potential

Zeta potential measurements were carried out using Zetasizer Nano 2S (Malvern Instruments, Ltd.) in order to determine the surface charge of the emulsion droplets. Samples were prepared by diluting emulsion samples in distilled water (0.32 g emulsion + 40 g of distilled water) and vortexed before measurement. Samples were placed in DTS-1070 disposable folded capillary cell (Malvern Instruments, Ltd., United Kingdom). The zeta potential range was set to -100 to +50 mV and measurements were done at 25°C on the samples collected on day 2. Measurements were done in duplicates.

2.4.6. Apparent viscosity

Apparent viscosity was determined with a stress-controlled rheometer (Stresstech, Reologica Instruments AB, Lund, Sweden) on days 1 and 12. CC25 standard bob cup system in a temperature vessel was used in order to perform the measurements. Emulsions (15 ml) were measured over a shear stress range from 0.0125 to 50 Pa at 25°C. Results were calculated

on a specific shear rate (20 s^{-1}) for each emulsion in Pascal second (Pa·s). Samples were measured in duplicate.

2.5. Methods for lipid oxidation measurements of emulsions

2.5.1. Primary oxidation products—peroxide value (PV)

Primary oxidation products were determined according to the Bligh and Dyer method with slight changes (Bligh & Dyer, 1959). Lipids were extracted using 5 g of emulsion and a reduced amount of solvent (30.0 mL of methanol and chloroform, 1:1). PV was subsequently determined on the lipid extracts by colorimetric determination of iron thiocyanate on a spectrophotometer (Shimadzu, UV mini 1240, Kyoto, Japan) at 500 nm (Shantha and Decker, 1994). Measurements were done in duplicates.

2.5.2. Tocopherol content - HPLC

Tocopherol content of emulsions was analyzed by HPLC (Agilent 1100 Series; Column: Waters Spherisorb 3 µm Silica; 4.6×150 mm). Tocopherol analysis was performed according to the official AOCS method (1998) using lipid extracts (Section 2.5.1), which were further evaporated and dissolved in heptane. Measurements were carried out in duplicates.

2.5.3. Secondary oxidation products—Dynamic Head Space GC-MS

Volatile secondary oxidation products were analyzed according to the method described by Yesiltas et al (2018a). Volatile compounds were trapped on Tenax GR tubes. The volatile compounds were separated in a gas chromatograph (Agilent Technologies, 6890N Network GC System, DE, USA) on a 30 m DB 1701 fused silica capillary column (0.25 mm i.d., 1 µm

film thickness; Agilent Technologies, J&W GC Columns, CA, USA). Mass-spectrometry (Agilent 5973 Network Mass Selective Detector, Agilent Technologies, 70 eV; mass to charge ratio scan between 30 and 250) was used to analyze individual volatile compounds and MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard) was used for the identification. The volatile compounds 2-ethyl-furan, 1-penten-3-one, 1-penten-3-ol, *(E)*-2-pentenal, hexanal, *(E)*-2-hexenal, *(Z)*-4-heptenal, 2-pentyl-furan, *(E)*-2-heptenal, benzaldehyde, *(E,E)*-2,4-heptadienal, nonanal and *(E,Z)*-2,6-nonadienal were analyzed in emulsion samples.

2.6. Statistical analysis and principle component analysis (PCA)

Statgraphics XVII (Statpoint Technologies, Inc., Virginia, USA) was used to carry out the analysis of variance (ANOVA) using Fisher's least significant difference test. The significance was evaluated statistically at the confidence level 1-α = 95%.

PCA was performed using Latentix 2.12 (LatentiX, Copenhagen, Denmark). It was carried out with the emulsions as objects and creaming, droplet size, zeta potential, viscosity, peroxide value, alpha-tocopherol, and volatile compounds as variables. The data was transformed using the autoscale function in Latentix and the PCA models were calculated.

3. Results and Discussion

3.1. PCA of all emulsions

Two different PCA models were calculated; one where all time points for each of the variables were included and one where only the last time point (day 12) of the variables was included. The conclusion that could be made from these 2 models was more or less the same. For the

sake of simplicity, only the model calculated with the last time point for the variables is shown in Fig. 1. The first principle component (PC1) and second principle component (PC2) explained 43 and 29% of the variation in the data, respectively. The scores plot showed that the 1_CAS emulsion located differently compared to the rest of the emulsions, indicating that it behaved differently with respect to the physical and oxidative parameters measured. The loadings plot showed that the location of CAS was mostly explained by the high viscosity as well as higher protein content in the aqueous phase (non-adsorbed CAS). Oxidation parameters, PV, tocopherols and volatile compounds were located in the same quadrant and correlated with the following emulsions: 2_PC_com, 3_PC_C14_360, 6_PC_C16_360, and 9_PC_com_caf_360, which were most prone to lipid oxidation.

Emulsion 10_PC_com_caf_2160 was mostly explained by less negative zeta potential, high creaming, low viscosity and low non-adsorbed proteins. Emulsions produced with middle and high concentrations of modified PCs were not described specifically by any of these physical parameters. However, they located far from the oxidative parameters, which indicated that their oxidative stability were superior compared to the rest. In order to obtain a better overall picture of the emulsions according to the physical parameters, see the biplot (Supplementary Fig. 2) for the PCA model calculated only with physical parameters.

It was observed that the emulsions with PC_C14 were closer to viscosity and non-adsorbed CAS compared to PC_C16, whereas emulsions with PC_C16 located closer to zeta potential and creaming. This indicated differences in physical stability. Thus, emulsions containing PC_C14 had higher viscosity, which might be due to the higher amount of non-adsorbed CAS content in the water phase. On the other hand, emulsions containing PC_C16 had higher

292 protein surface load, creaming, and lower negative zeta potential. Moreover, the oxidation
293 parameters showed that the emulsions with PC_C14 located closer to oxidation parameters
294 (volatiles, PV) compared to PC_C16 emulsions, which confirmed better oxidative stability of
295 the emulsions produced with PC_C16. In order to confirm this interpretation of the PCA
296 model, the original raw data of the physical and oxidative stability parameters were studied in
297 more detail.

298 ## 3.2. Characterization of emulsions

299 ### 3.2.1. Creaming Index

300 Emulsions produced with the combinations of CAS and soy PC were creamed 4-6%, whereas
301 emulsions produced with only CAS did not have any creaming during 12 days of storage
302 (Supplementary Fig. 3a,b). Modified emulsifiers also showed between 2-4% creaming during
303 12 days of storage. As these emulsions have lower creaming rate than 1 mm/day, they were
304 all considered as stable emulsions (McClements, 1999).

305 ### 3.2.2. Interfacial tension of the emulsifier combinations

306 Oil-water interfacial tension of the aqueous phase of the emulsions was measured in order to
307 determine the effects of substituting 45% of the CAS with different PCs. The interfacial
308 tension values of all the samples at 15 min were significantly different from each other
309 ($p<0.05$). It was observed that 2_PC_com had an oil-water interfacial tension of 11.47 ± 0.35
310 mN/m, whereas aqueous phase of 1_CAS had an interfacial tension of 8.34 ± 0.02 mN/m
311 (Fig. 2). Thus, including soy PC increased the interfacial tension. However, when 60% of the
312 soy PC in 2_PC_com was substituted with PC_C14 or PC_16, interfacial tension dropped

significantly from 11.47 ± 0.35 mN/m to 4.57 ± 0.01 or 3.94 ± 0.04 mN/m, respectively. Thus, although both commercial and modified PC's could self-assemble in the aqueous phase and form micelles, significant differences in interfacial tension values were observed between the different emulsifier combinations. Aqueous phases with PC_C16 showed significantly lower interfacial tension compared to PC_C14, which could be due to the interaction of PC_C16 with soy PC and CAS at the interface as well as higher affinity to the oil phase. Low interfacial tension provided by modified PCs implies a superior surface activity, which provided minimized contact area between hydrophobic and hydrophilic regions.

3.2.3. Droplet size

The mean particle diameter (D[4,3]) of the emulsions was determined in order to observe the impact of emulsifier types and different concentrations of modified PCs (Table 2). Substituting 45% of CAS with soy PC resulted in a significant increase in droplet size from 8.8 ± 0.3 to 17 ± 0.2 µm. However, increasing concentration of modified PCs in the emulsions led to a significant decrease in droplet size (Table 2). This could be due to the fact that modified PCs had one alkyl chain instead of 2 alkyl chains compared to soy PC as well as a larger head part as a result of covalently attached caffeic acid in the glycerol backbone, which might have enhanced the surface activity. This was valid for both PC_C14 and PC_C16, which could be attributed to their fast adsorption rates compared to proteins and soy PC, thereby producing smaller oil droplets by inhibiting droplet coalescence during homogenization. Improved performance of lysolecithins compared to conventional PC with 2 fatty acid chains was reported previously by other researchers (Choi et al., 2011; Casado, Martin, Torres, & Reglero, 2012). This could be due to higher HLB values of modified PCs compared to

conventional PC (HLB = 8), which provides higher surface activity and better stabilizing ability in oil-in-water emulsions. It has been emphasized that lysolecithins usually disperse better in the aqueous phase which influence their effectiveness (McClements et al, 2017).

When the droplet size of the emulsions with the equivalent concentrations of modified PCs was compared, it was observed that PC_C14 provided smaller droplets compared to PC_C16, which indicated faster adsorption of PC_C14 at the oil-water interface compared to PC_C16 at their middle and higher concentrations. At the low concentration of modified PCs, there was no significant effect of chain length observed for droplet sizes.

These results are generally consistent with the interfacial tension results, for which it was found that the aqueous phase of 2_PC_com had higher oil-water interfacial tension compared to 1_CAS, whereas addition of modified PCs decreased the interfacial tension significantly. Higher surface activity of modified PCs improved emulsification of smaller droplets before their coalescence. Moreover, it was obvious that the increasing concentration of modified PCs provided significant decrease in droplet size (Table 2). The change in the droplet size with increased concentration of modified PCs can also be seen in the optical microscope images (Supplementary Fig. 4a,b). There was no significant increase in D[4,3] droplet sizes observed during storage except for 5_PC_C14_2160 (Table 2, Supplementary Fig. 5a).

3.2.4. Protein content in the aqueous phase and the surface load of proteins

Protein content in the aqueous phase has several effects on physical and oxidative stability of the emulsions. A previous study showed that viscosity of the emulsions was directly increased by the concentration of CAS in the continuous water phase when the concentration was

between 5-10% in 30% oil-in-water emulsion (Liang et al., 2014). Viscosity of the final emulsion increases with the increasing viscosity of the continuous water phase (Tesch & Schubert, 2002; Yesiltas et al., 2018a). It was observed that the protein content in the water phase was around 2-fold higher for emulsion produced with only CAS compared to the rest of the emulsions, which might explain the significantly higher viscosity of the CAS emulsion (Table 1). On the other hand, proteins (e.g., CAS) inhibit oxidation due to their radical scavenging and metal chelating activities, which allow proteins to deactivate prooxidants either at the oil-water interface or in the aqueous phase (Faraji, McClements, & Decker, 2004; Berton, Ropers, Viau, & Genot, 2011). For these reasons, we have determined the protein content in the aqueous phases of the emulsions. Additionally, we have calculated the protein surface load in order to indirectly infer the adsorption of modified PCs at the interface. Forty to 66% of the protein was non-adsorbed in the aqueous phase, which indicated that the proteins were available in the aqueous phase in concentrations ranging between 2.25 – 6.23 wt%. Non-adsorbed protein of 8_PC_C16_2160 was significantly lower compared to the rest of the emulsions except for 6_PC_C16_1080 and 7_PC_C16_2160 (Table 2). Changing modified PC concentration did not affect the protein content in the aqueous phase significantly neither for PC_C14 nor PC_C16. Given that 5_PC_C14_2160 had significantly more protein content in the water phase compared to 8_PC_C16_2160, PC_C14 showed better surface activity at the oil-water interface, thereby replacing more protein at the oil-water interface. Protein surface load results revealed more information about the adsorbed protein due to the impact of droplet size on the protein surface load. Interestingly, involvement of soy PC led to 4 folds higher protein load at the oil-water interface compared to 1_CAS (see 1_CAS versus 2_PC_com, 9_PC_com_caf_360, and 10_PC_com_caf_2160, Table 2). This could be due to

379 the interaction between CAS and soy PC, which presumably resulted in the adsorption of
380 larger CAS aggregates and PC multilayers at the interface. This hypothesis was supported by
381 results from another study from our lab, which indicated that emulsions produced with CAS
382 and PC provided thicker interface layer compared to only CAS emulsions and which provided
383 a model for the complex structure of 70% oil-in-water emulsions with CAS and PC as
384 emulsifiers (data submitted for publication). Protein surface load decreased with increasing
385 concentration of modified PCs both for PC_C14 and PC_C16 (Table 2). Emulsions with
386 PC_C14 had lower protein surface load compared to emulsions with PC_C16, which would
387 normally support that PC_C14 was more surface active and replaced more proteins at the
388 interface compared to PC_C16. However, interfacial tension results (Section 3.1.2) indicated
389 that aqueous phase with PC_C16 had lower interfacial tension (4 mN/m) compared to
390 PC_C14 (5 mN/m). This was presumably due to PC_C16`s more balanced molecular
391 interactions with soy PC and CAS at the interface, such as forming a densely packed
392 interfacial layer with less permeability, which are not merely related to the surface activity of
393 the molecule. Therefore, these results could be attributed to thicker oil-water interfacial layer
394 formed in the presence of PC_C16, which thereby led to a higher protein surface load
395 compared to PC_C14.

396 **3.2.5. Zeta potential**

397 All the samples had negative zeta potential, which was mainly attributed to the CAS being
398 above its isoelectric point (pH 4.6) as the pH of the emulsion was 7 (O'Kennedy, 2011) (Table
399 2). Soy PC is a zwitterionic molecule, but it generally gives negative surface charge at neutral
400 conditions (Anankanbil et al., 2018). Modified PCs were also negatively charged due to the

phosphate groups at pH 7. Thus, when these emulsifiers were used alone to emulsify 20% fish oil-in-water emulsions at pH 7, they resulted in a surface charge of -37.2 ± 0.2, -39.5 ± 0.1 and -50.2 ± 0.1 mV for PC_C14, PC_C16 and soy PC, respectively (Anankanbil et al., 2018). In the current study, it was observed that the substitution of some of the CAS with soy PC or soy PC + modified PCs resulted in less negatively charged lipid droplets.

Free caffeic acid addition did not result in any significant change in surface charge of the emulsions produced with CAS and soy PC (Table 2). Increasing the concentration of modified PCs did not have a clear effect on surface charge. Comparison of the surface charge of the emulsions produced with equivalent concentrations (360 and 1080 ppm caffeic acid) of modified PCs showed that the emulsions with PC_C14 provided more negatively charged particles compared to PC_C16. The determination of the protein content in the aqueous phase and protein load at the interface showed that PC_16 interacted more with CAS and soy PC at the oil-water interface compared to PC_C14. Since CAS is more negatively charged than modified PCs, one would expect that the PC_16 emulsions would have a more negative charge than the emulsion with PC_14. However, adsorption of these emulsifiers does not necessarily result in a monolayer. The overall zeta potential of the oil droplets will be affected by all the emulsifiers involved, e.g., CAS, commercial PC and modified PC as well as their conformation at the oil-water interface depending on their interactions, which might not lead to a straight forward correlation between zeta potential results and emulsifiers used. Therefore, protein surface load results indicated that the interaction between CAS, soy PC and PC_C16 resulted in a thicker interfacial layer, which gave less negative zeta potential due to the higher amount of PC_C16 compared to the amount of PC_C14 when involved in the emulsions.

The reason for less negatively charged droplet surfaces for 2_PC_com compared to 3_PC_C14_360 and more negatively charged than 7_PC_C16_1080 could also be attributed to higher amount of CAS, soy PC and PC_C16 adsorbed at the oil-water interface and the interaction between emulsifiers forming a thicker interface. Moreover, as there was no significant difference in protein surface load of emulsions produced with PC_C14 and PC_C16 at their equivalent concentrations, different surface charge could be attributed to the content of soy PC and modified PCs. As emulsions with PC_C16 had less negative zeta potential, this indicates the presence of higher amount of soy PC and PC_C16 at the oil-water interface compared to emulsions produced with PC_C14.

3.2.6. Apparent viscosity

Emulsions were non-Newtonian and showed shear thinning behavior. Substitution of 45% of the CAS with soy PC decreased viscosity significantly as expected due to the increase in droplet size (D[4,3], Table 2). Viscosity of the emulsions decreased significantly during storage except for 1_CAS and 9_PC_com_caf_360 (Table 2, Supplementary Fig. 5b). Addition of 2160 ppm free caffeic acid into the CAS + soy PC emulsions resulted in significant decrease in the viscosity as well, which correlated well with the significant increase in the droplet size (D[4,3], Table 2). This negative correlation between droplet size and viscosity could be due to smaller droplets leading to more friction between oil droplets at an expanded surface-to-volume ratio of the dispersed phase. This results in less mobility of the droplets in the emulsion and therefore provides higher viscosity compared to emulsions having larger droplets (Yesiltas et al., 2019). Similar observations regarding decrease in viscosity with the

addition of caffeic acid were made for 70% fish oil-in-water emulsions produced with CAS and diacetyl tartaric acid esters of mono- and diglycerides (DATEM) (Yesiltas et al., 2018b).

On the other hand, this argument was not valid when looking at the effects of increased modified PCs concentration on viscosity. It was observed that the viscosity did not necessarily increase with decreasing droplet size. This could be due to the effect of protein content in the aqueous phase. As discussed in section 3.1.4, protein content in the aqueous phase might increase the viscosity of the final emulsion when proteins are present in higher concentrations. The concentration of CAS in the aqueous phase (2.25 – 6.23 wt%, Table 2) might have affected the viscosity of the emulsions with modified PCs at their highest and middle concentrations; 4_PC_C14_1080 and 5_PC_C14_2160 had significantly higher viscosity compared to 7_PC_C16_1080 and 8_PC_C16_2160, respectively, whereas this was not the case for the lower concentrations of modified PCs (3_PC_C14_360 and 6_PC_C16_360).

3.3. Oxidative stability of emulsions

3.3.1. Primary oxidation products - peroxide value

Lipid hydroperoxides were analyzed to compare the oxidation rate of the emulsions during 12 days of storage (Fig. 3). As expected the use of modified PCs' had an impact on the oxidative stability of high fat fish oil-in-water emulsions. Peroxide value (PV) of 1_CAS was lower compared to 2_PC_com, which showed that substitution of 45% of the CAS with soy PC did not improve the oxidative stability of the 70% fish oil-in-water emulsion in terms of primary oxidation products. The addition of modified PCs low concentrations (see 3_PC_C14_360

and 6_PC_C16_360, in Fig. 3) resulted in prooxidant effect when compared to 2_PC_com. Emulsions containing modified PCs had higher PV than CAS + soy PC emulsions, which contained the equivalent amount of caffeic acid but in free form. Thus, the use of modified PC with caffeic acid attached did not reduce the formation of hydroperoxides when added both at low and high concentrations. The beneficial effect of having free caffeic acid in the aqueous phase compared to having caffeic acid covalently attached to the PC could be due to the enhanced ability of free caffeic acid in binding iron in the aqueous phase, which limits metal catalyzed initiation and free radical formation (Frankel, 2012a).

Emulsions with PC_C16 had higher PV compared to emulsions with equivalent amount of PC_C14. This could be explained by the faster degradation of hydroperoxides in emulsions with PC_C14 (see section 3.3.3).

3.3.2. Changes in tocopherol content

Alpha-, gamma-, and delta-tocopherol levels in emulsions were in the range of 105.7 ± 0.7 – 133.6 ± 3.7, 72.1 ± 0.1 – 74.2 ± 0.7, 29.1 ± 0.7 – 30.5 ± 0.3 µg toc /g emulsion at day 0 (Supplementary Fig. 6a-c). As the fish oil content was the same in all emulsions, significant differences between tocopherol contents in emulsions were attributed to the consumption of tocopherol during emulsion production at day 0. Emulsion 1_CAS had significantly higher consumption of all types of tocopherols compared to 2_PC_com. When the soy PC was substituted with increasing concentration of modified PCs, alpha tocopherol content decreased significantly, which indicated that the alpha-tocopherols were acting as antioxidants in the presence of modified PCs or there was an interaction between modified

PCs and alpha-tocopherols during emulsification. Free caffeic acid addition did not have any significant effect on any tocopherols in the emulsions produced with CAS and soy PC, which indicated that the alpha-tocopherol content was not affected by the presence of free caffeic acid at day 0.

The changes in the tocopherol content were followed during 12 days of storage and it was found that the alpha-tocopherol content decreased significantly only for 5_PC_C14_2160 and 8_PC_C16_2160, whereas gamma-tocopherol decreased significantly for 5_PC_C14_2160, 8_PC_C16_2160, and 10_PC_com_caf_2160 (Supplementary Fig. 6a,c). There was no significant decrease in delta-tocopherol content of the emulsions during 12 days of storage. These results indicated that the total tocopherol content of the emulsions with the highest concentration of caffeic acid decreased significantly, which showed that part of the antioxidant activity was due to tocopherols in these emulsions. However, the decrease in the amount of tocopherol from day 0 to 12 was only around 5μg toc/g sample. It should also be considered that alpha-tocopherols can be regenerated from oxidized tocopherol (e.g., tocopherol quinone) with the proton-donating capacity of the amino group of phospholipids such as phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (García-Moreno et al., 2014; Samdani, McClements, & Decker, 2018). Therefore, the antioxidant activity of alpha-tocopherol could be even higher than what was measured due to its possible consumption and regeneration.

3.3.3. Secondary volatile oxidation products – DHS GC-MS

Volatile oxidation products formed in the emulsions showed similar trend in terms of their content during 12 days of storage; therefore, sum of the volatiles was presented in Fig. 4. Development of 1-penten-3-ol, *(E,E)*-2,4-heptadienal, 2-pentenal, and 2-ethylfuran, which were in high concentration compared to rest of the volatile compounds, are also shown in Supplementary Fig. 7a-d. It was observed that 360 ppm caffeic acid provided with modified PCs resulted in a prooxidant effect. On the contrary, physical mixture of caffeic acid and PC (9_PC_com_caf_360) did not cause a prooxidant effect, when compared to 2_PC_com.

For both modified PCs, oxidative stability was improved with the increasing amount of modified PCs. All emulsions with modified PC added in concentrations above 360 mg/kg had lower formation of volatile oxidation products than the emulsion with commercial PC as also observed in the PCA plot. Improved oxidative stability of emulsions with modified PCs could be due to the fact that caffeic acid was located at the interface showing antioxidant activity. It could also be due to the larger surfactant head group of modified PCs compared to soy PC, which led to thicker oil-water interfacial layer (Berton-Carabin et al., 2014).

Emulsion 8_PC_C16_2160 had better oxidative stability compared to 10_PC_com_caf_2160, which showed that caffeic acid in high concentrations was more efficient when attached to PC_16 than when present in its free form. This could be attributed to different interface structure and composition of the emulsions due to the molecular structure differences between PC and PC_16, which affects the adsorption performance of the molecules and the location of caffeic acid. It is worth paying attention to the significant differences in physical properties of these two emulsions such as droplet size. There has been contradicting results on the impact of droplet size on oxidative stability (Berton-Carabin et al., 2014). Nevertheless,

studies which have shown an impact of droplet size have found that small droplets increase lipid oxidation. Hence, the large droplets in the soy PC emulsions would favor decreased oxidation and not increased oxidation as observed here.

Results also showed that on the last day of storage, CAS emulsion had higher concentrations of volatile compound than emulsions with PC_C14_2160, PC_C16_1080 and PC_C16_2160 ppm.

On the other hand, PC_C16 had a lower amount of volatile compounds formed compared to PC_C14 in all concentrations of added modified PCs (Fig. 3), which could be attributed to the interface structure of the emulsions. This could be explained by the interfacial tension results discussed under the section 3.2.2, where PC_C16 was indicated to have a better interaction with CAS and soy PC, thereby forming a thicker interface, higher packing density and less permeability at the oil-water interface compared to PC_C14 (McClements and Decker, 2018). Moreover, as it was discussed in the section 3.2.4, PC_C16 had lower amount of non-adsorbed protein in the aqueous phase compared to PC_C14 at their highest concentration, thereby higher protein surface load, which supported the formation of a thicker interfacial layer for PC_C16 and a better coverage of the oil droplets compared to PC_C14. Higher amount of emulsifiers at the oil-water interface inhibited prooxidant diffusion from aqueous phase to oil phase and brought antioxidant activity (due to the presence of antioxidant emulsifiers) to the interface, thereby increasing oxidative stability.

Another reason for higher oxidative stability could be attributed to the diffusion of some of the PC_C16 into the oil phase in low concentrations (e.g., below its critical micelle concentration),

549 due to its high hydrophobicity and thereby acting as a chain-breaking antioxidant in the oil
550 phase. Individual PC_C16 molecules might act as an antioxidant owing to the covalently
551 attached caffeic acid on the head group of the molecule and thereby contribute to the overall
552 increased oxidative stability of the emulsions with PC_C16. Besides, both modified PCs could
553 also have potential hydrophobic interactions with emulsifiers or aggregates in the aqueous
554 phase, which might have improved their antioxidative effects (Shahidi and Zhong, 2011).

555 **4. Conclusion**

556 Soy and modified PCs in combination with CAS, as emulsifiers, decreased the viscosity of
557 70% omega-3 delivery emulsions compared to emulsions stabilized with only CAS. This
558 suggests that the emulsions can potentially be used in wider range of applications in food
559 systems when higher amount of omega-3 polyunsaturated fatty acids are needed. The use of
560 soy PC in combination with CAS decreased physical stability. However, the physical stability
561 was significantly improved when soy PC was partly replaced by modified PCs with C14 or
562 C16 alkyl chain as well as covalently attached caffeic acid due to their high surface activity.
563 The highest oxidative stability was observed for the emulsion with the highest concentration of
564 modified PC with C16 alkyl chain length and covalently attached caffeic acid. This was
565 attributed to its low interfacial tension and ability to interact with CAS and soy PC, which led to
566 thicker interfacial layer and less permeability for prooxidants, thereby resulting in better
567 oxidative stability.

568 **Abbreviations used**

569 **CAS** – sodium caseinate

DHA – docosohexaenoic acid

DHS – dynamic head space

EPA – eicosapentaenoic acid

LC n-3 PUFAs – Long chain omega-3 polyunsaturated fatty acid

PC – phosphatidylcholine

PC_C14 – modified PC with covalently attached caffeic acid and C14 alkyl chain

PC_C16 – modified PC with covalently attached caffeic acid and C16 alkyl chain

PCA – principle component analysis

PV – peroxide value

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Table 1. Emulsion codes, descriptions and experimental design with the content of emulsions

Emulsion code	Description	Soy PC (%, w/w)	Modified PC (%, w/w)	caffeic acid (ppm)
1 CAS	CAS only	-	-	-
2 PC_com	CAS + com PC	1.27	-	-
3 PC_C14_360	CAS + com PC + mod PC C14	1.15	0.12	360*
4 PC_C14_1080	CAS + com PC + mod PC C14	0.90	0.37	1080*
5 PC_C14_2160	CAS + com PC + mod PC C14	0.52	0.75	2160*
6 PC_C16_360	CAS + com PC + mod PC C16	1.14	0.13	360*
7 PC_C16_1080	CAS + com PC + mod PC C16	0.88	0.39	1080*
8 PC_C16_2160	CAS + com PC + mod PC C16	0.48	0.79	2160*
9 PC_com_caf_360	CAS + com PC + caffeic acid	1.27	-	360
10 PC_com_caf_2160	CAS + com PC + caffeic acid	1.27	-	2160

All the emulsions have 70% (w/w) fish oil, 2.8% (w/w) total emulsifier content and the ratio between CAS to PC is 1.2, which results in 1.53% (w/w) CAS, except for 1CAS as it includes only CAS (2.8%, w/w). Soy PC was substituted with modified PCs in different ratios in order to obtain different concentrations of caffeic acid in the final emulsion (caffeic acid (ppm)).

*Free caffeic acid was not added in emulsion 3 to 8. Concentration was calculated according to the added modified PC, which had caffeic acid attached to the compound itself.

Table 2. Droplet size, apparent viscosity, zeta potential, protein in the aqueous phase, and protein surface load results of emulsions

Emulsion code	D[3,2] (μm) (Day 1)	D[4,3] (μm) (Day 1)	Apparent viscosity (mPa·s) at 20 s^{-1} (Day 1)	Zeta potential (mV) (Day 2)	Protein in the aqueous phase (g) (Day 4)	Protein surface load (mg/m^2) (Day 4)
1 CAS	1.5 ± 0.7^{b}	8.8 ± 0.3^{d}	2457 ± 11^{d}	(-) 61.9 ± 1.4^{a}	1.70 ± 0.01^{c}	4.35 ± 0.05^{a}
2 PC_com	10.8 ± 0.2^{d}	17.0 ± 0.2^{g}	923 ± 14^{c}*	(-) 52.8 ± 4.4^{cd}	0.95 ± 0.34^{b}	16.47 ± 9.67^{b}
3 PC_C14_360	8.3 ± 0.1^{c}	11.7 ± 0.0^{f}	939 ± 7^{c}*	(-) 59.3 ± 5.2^{ab}	0.94 ± 0.05^{b}	12.81 ± 1.13^{b}
4 PC_C14_1080	1.0 ± 0.0^{ab}	8.0 ± 0.1^{c}	841 ± 9^{b}*	(-) 56.3 ± 1.4^{bc}	0.91 ± 0.01^{b}	1.63 ± 0.01^{a}
5 PC_C14_2160	0.7 ± 0.1^{a}*	5.0 ± 0.1^{a}*	971 ± 3^{c}*	(-) 52.2 ± 1.1^{cd}	0.91 ± 0.01^{b}	1.13 ± 0.02^{a}
6 PC_C16_360	8.0 ± 0.1^{c}*	11.9 ± 0.0^{f}	943 ± 36^{c}*	(-) 52.1 ± 1.2^{cd}	0.82 ± 0.10^{ab}	14.80 ± 2.05^{b}
7 PC_C16_1080	1.8 ± 0.3§	9.5 ± 0.2^{e}*	740 ± 32^{a}*	(-) 47.0 ± 3.9^{e}	0.81 ± 0.06^{ab}	3.40 ± 0.30^{a}
8 PC_C16_2160	1.1 ± 0.3§	6.7 ± 0.3^{b}	777 ± 83^{ab}*	(-) 48.8 ± 1.3^{de}	0.61 ± 0.19^{a}	2.64 ± 0.54^{a}
9 PC_com_caf_360	11.1 ± 0.2^{d}	17.8 ± 0.1^{h}	844 ± 44^{b}*	(-) 48.8 ± 1.9^{de}	0.96 ± 0.01^{b}	16.59 ± 0.17^{b}
10 PC_com_caf_2160	10.9 ± 0.6^{d}	18.4 ± 0.0^{i}	831 ± 93^{b}*	(-) 49.4 ± 3.0^{de}	1.00 ± 0.08^{b}	15.00 ± 2.29^{b}

*Significant changes happened in droplet size and viscosity during 12 days of storage at $p<0.05$.

$^{a-f}$Letters indicate the significant differences between samples for the same physical parameter.

§There was only one replicate for these 2 samples.

Figure 1

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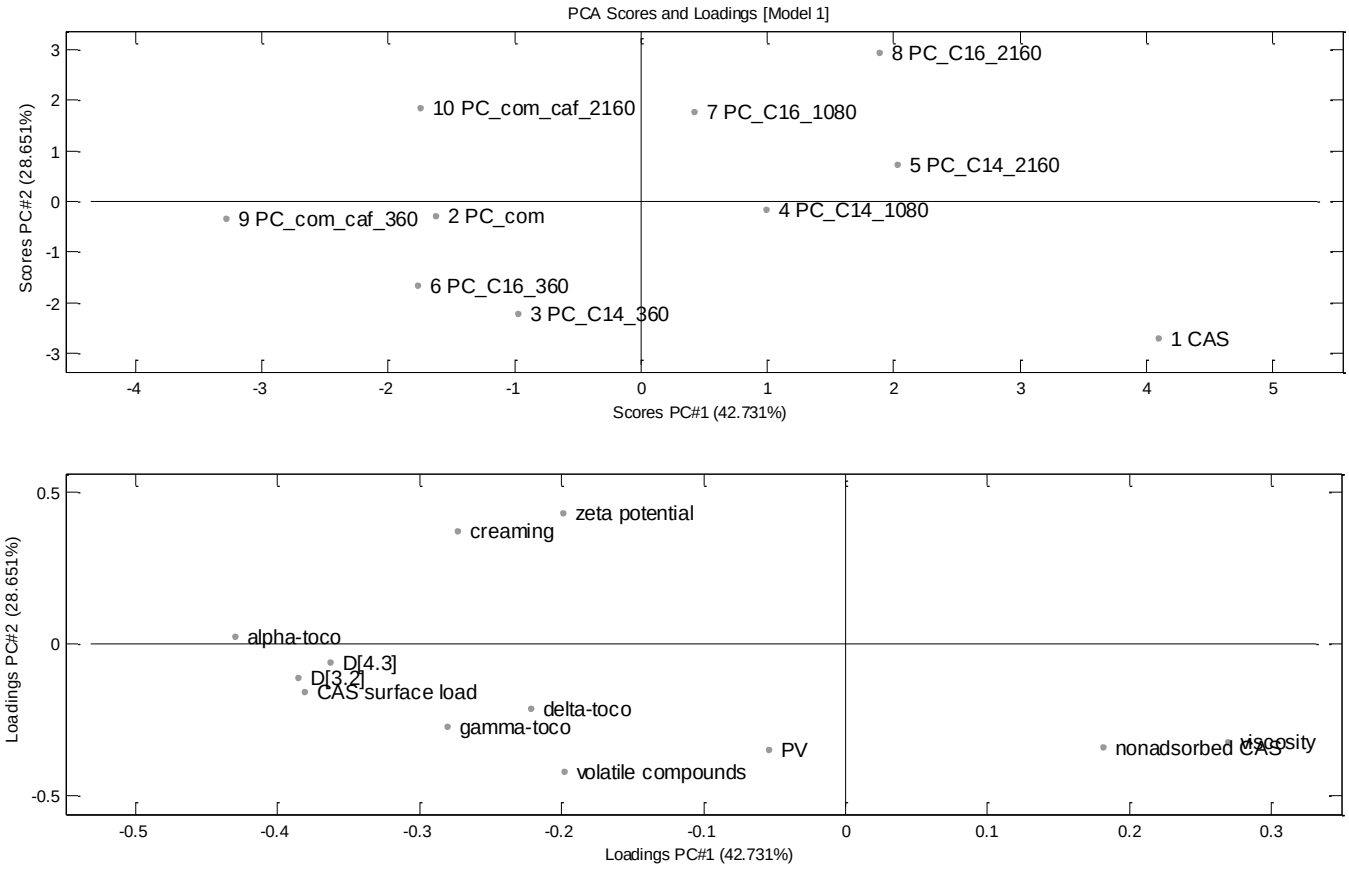
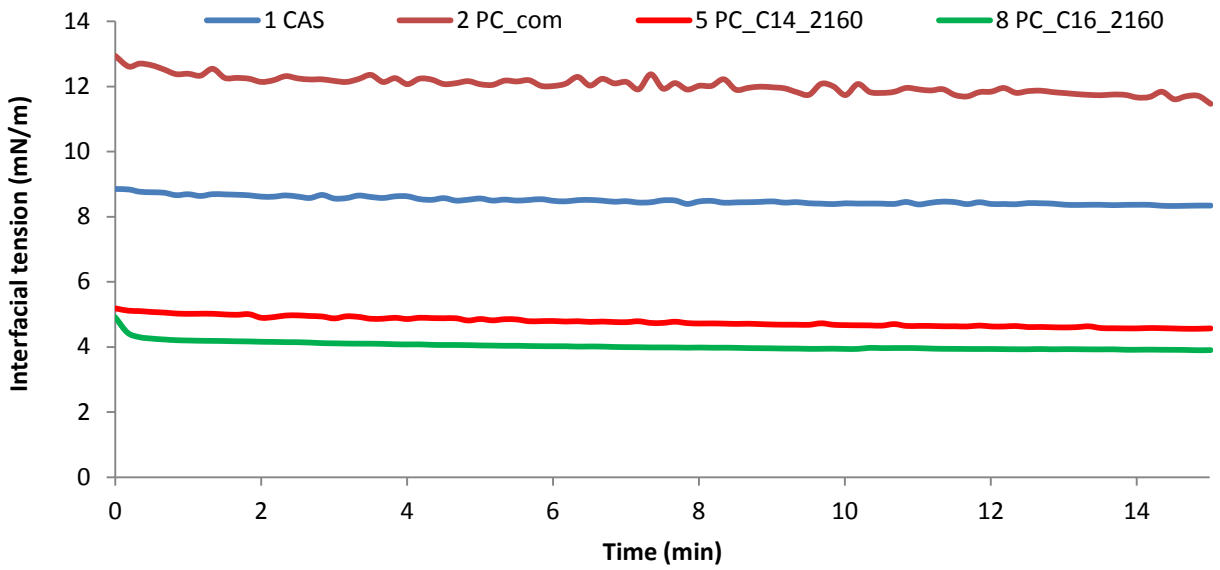


Figure 1. PCA scores and loading were plotted using the results of oxidation parameters (PV, tocopherols, volatiles) and physical parameters (D[3,2], D[4,3], viscosity, zeta potential, creaming, non-adsorbed CAS and CAS surface load).

Figure 2

Click here to download Figure(s): Figure 2.docx



The oil-water interfacial tension without emulsifiers was 26 mN/m during 15 min for MCT oil/water. Relative standard deviation was lower than 6% in all samples.

Figure 2. Interfacial tension of emulsifier combinations

Figure 3

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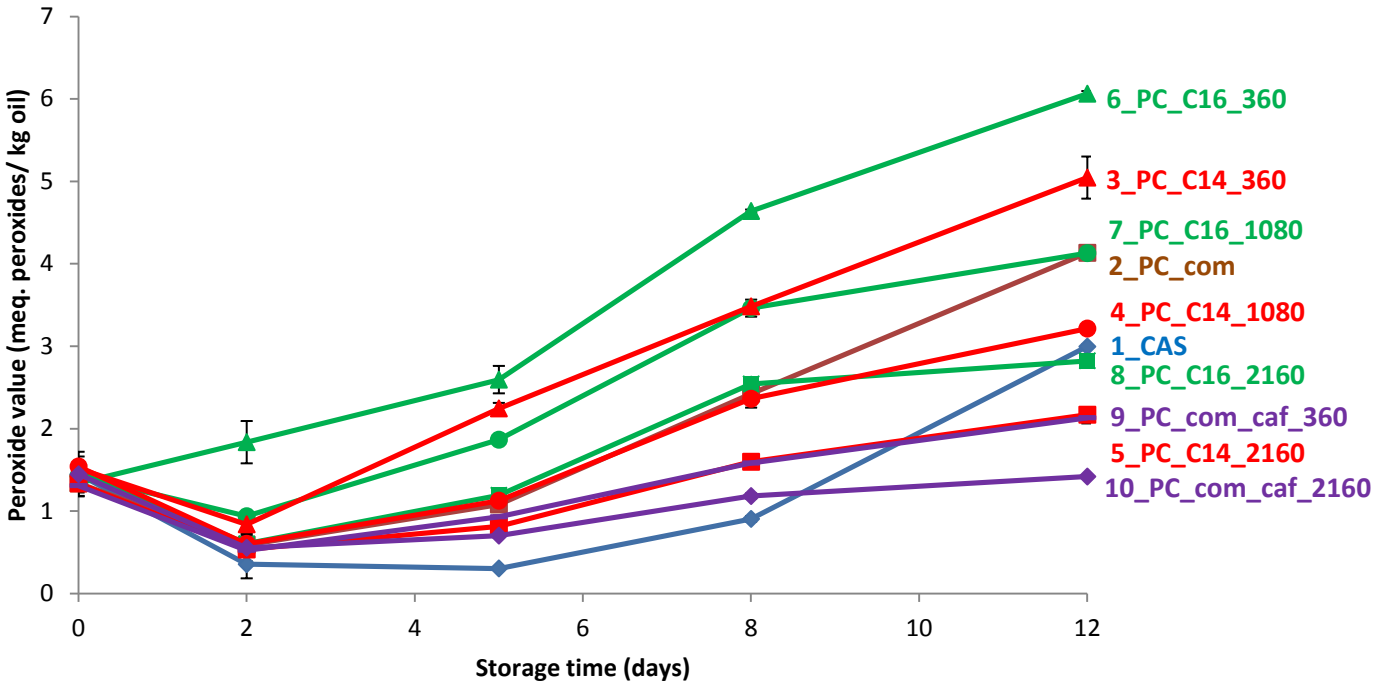

Figure 3. Formation of primary oxidation product in the emulsions during 12 days of storage.

Figure 4

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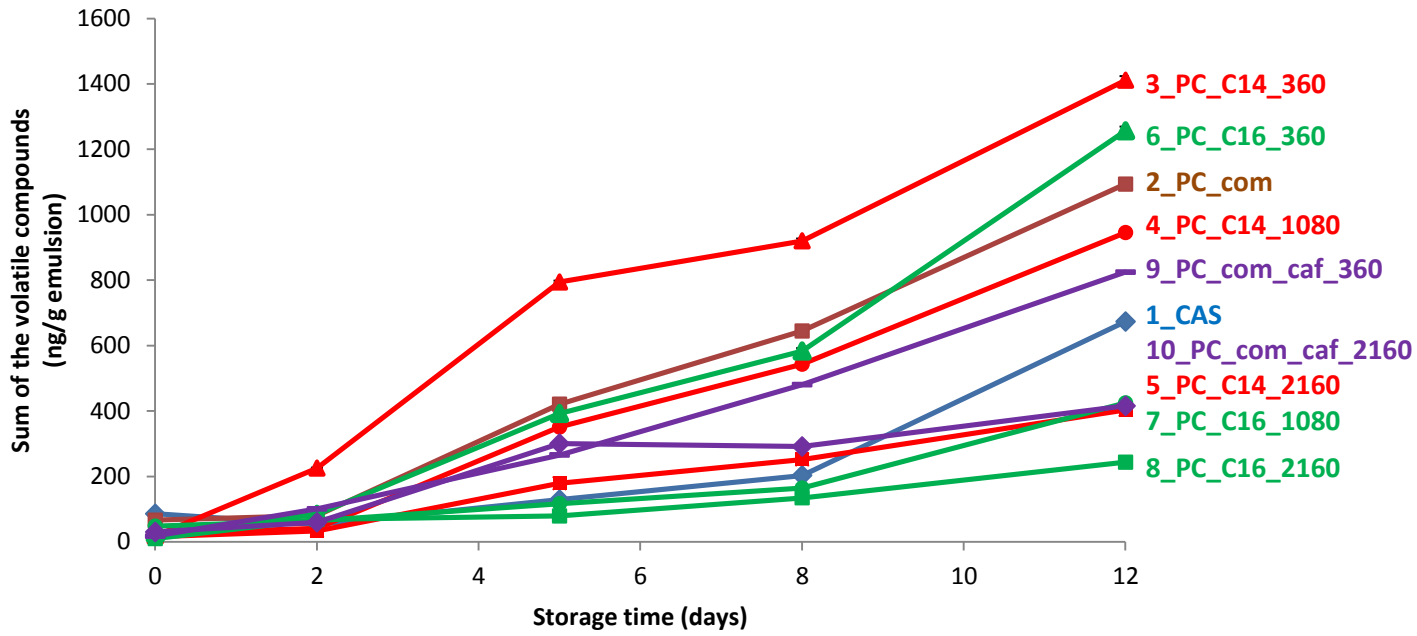

Figure 4. Sum of the volatile secondary oxidation products formed in emulsion samples during 12 days of storage

Supplementary Material

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