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The Use of Soy and Egg Phosphatidylcholines Modified with Caffeic Acid Enhances the Oxidative Stability of High-Fat (70%) Fish Oil-in-Water Emulsions

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Abstract: This study investigated the effect of the combined use of sodium caseinate (CAS), commercial phosphatidylcholine (PC), and modified PCs on the physical and oxidative stability of 70% fish oil-in-water emulsions. Caffeic acid was covalently attached to both modified PCs (PCs originated from soy and eggs) in order to increase the antioxidant activity of PCs and investigate the advantage of bringing the antioxidant activity to the close proximity of the oil-water interface. Results showed that oxidative stability was improved when part of the PC was substituted with modified soy PC or egg PC. Emulsions containing a low concentration of modified PCs (10 wt.% of total PC) resulted in a prooxidative effect on the formation of hydroperoxides compared to emulsions with free caffeic acid. On the other hand, a decrease in the formation of volatile oxidation products was observed for emulsions containing higher levels of modified PCs (60 wt.% of total PC) compared to the emulsions with free caffeic acid added at its equivalent concentration. Increased concentrations of modified PCs provided better oxidative stability in high-fat emulsions, independent of the modified PC type. Moreover, when oxidation was initiated by producing singlet oxygen near a single oil droplet using a focused laser, fluorescence imaging showed that the oxidation did not propagate from one oil droplet to another oil droplet.

Keywords: emulsifiers; surfactants; oxidation; oil-water interface; microscopy

Citation: Yesiltas, B.; García-Moreno, P.J.; Sørensen, A.-D.M.; Banerjee, C.; Anankanbil, S.; Guo, Z.; Ogilby, P.R.; Jacobsen, C. The Use of Soy and Egg Phosphatidylcholines Modified with Caffeic Acid Enhances the Oxidative Stability of High-Fat (70%) Fish Oil-in-Water Emulsions. *Colloids Interfaces* **2023**, *7*, 60. https://doi.org/10.3390/colloids7030060

Academic Editors: Eleni P. Kalogianni and Reinhard Miller

Received: 29 June 2023
Revised: 16 August 2023
Accepted: 12 September 2023
Published: 18 September 2023

1. Introduction

Omega-3 polyunsaturated fatty acids (PUFAs), particularly the long-chain eicosapentaenoic (EPA, C20:5n-3) and docosahexaenoic (DHA, C22:6n-3) fatty acids, have been reported to play a role in promoting health and providing protection against diseases [1]. Although EPA and DHA are not essential fatty acids, the conversion of α-linolenic acid to EPA and DHA is very low in humans [2]. Therefore, humans need to ingest EPA and DHA through the diet to benefit from their health effects, e.g., reduction in risk of cardiovascular diseases, improvements in visual and neurological development, and inflammatory conditions [2]. Nevertheless, Eastern societies have low consumption of seafood, which is the main source of omega-3 PUFAs [1]. For that reason, the food industry has gained interest in the development of generally consumed foods fortified with omega-3 PUFAs (e.g., milk, mayonnaise, and other dressings, among others) [3,4].

Omega-3 PUFAs are highly prone to lipid oxidation due to their high content of bis-allylic hydrogens in their structure, which makes their incorporation into food matrices challenging without reducing their nutritional and sensory properties [5,6]. Among the technological approaches investigated to successfully deliver omega-3 fatty acids, the

development of fish or algae oil-in-water emulsions is the most commonly employed for enriching water-based liquid or semi-liquid food matrices due to the ease of mixing [7]. Particularly interesting is the development of high-fat (>50%) oil-in-water emulsions, which enables the reduction of the amount of delivery system incorporated into the food matrix for a determined level of omega-3 enrichment. Moreover, high-fat emulsions are preferred for fortifying highly viscous foods (i.e., cream cheese and dressings) since they reduce the impact on the textural properties of the final product [8,9]. However, high-fat oil-in-water emulsions present a pronounced packing of oil droplets as a consequence of their high volume fraction in the dispersed phase. This makes it especially challenging to develop physically and oxidatively stable high-fish or algae (>50%) oil-in-water emulsions [10].

The interfacial properties of emulsions, which are determined by the type of emulsifier employed, are key factors influencing the physicochemical stability of these thermodynamically unstable systems [11]. Indeed, aiming for a location of antioxidants at the oil/water interface, which is the place where lipid oxidation is initiated in heterogenous systems, has been a widely studied strategy to improve the oxidative stability of emulsions [12]. Antioxidants can be placed at the oil/water interface by (1) lipophilization of hydrophilic antioxidants [13] or (2) using antioxidant emulsifiers, which might consist of existing emulsifiers exhibiting antioxidant activity (i.e., proteins, phospholipids) [14] or new emulsifiers formed by linking surface-active and antioxidant molecules [15,16].

Recently, we have reported the stabilization of high-fat emulsions with the combination of casein, a potent emulsifier leading to physically stable emulsions [17], and novel antioxidant emulsifiers such as commercial diacetyl tartaric acid esters of mono- and diglycerides (DATEM) or phosphatidylcholine extracted from soybean (soy-PC), both covalently modified with caffeic acids [18,19]. Both studies indicated that the location of caffeic acid at the interface when bonded to DATEM or PC enhanced the oxidative stability of emulsions when compared to the addition of free caffeic acid or the use of unmodified emulsifiers. The particular significance is the design of bioinspired interfaces consisting of combinations of proteins and phospholipids, which have been suggested to provide better stabilization of oil-in-water emulsions compared to single emulsifiers [14,20,21]. However, the effect of the phospholipid source, which determines the fatty acid composition and thus their susceptibility to oxidation, on the physicochemical stabilization of high-fat oil-in-water emulsions, remains to be investigated.

Thus, this work aimed to investigate the use of soybean and egg PCs modified with caffeic acid for the stabilization of high-fat fish oil-in-water emulsions in combination with casein. Specifically, we investigated the interaction of modified PC with commercial PC and sodium caseinate in the emulsion and the effect of bringing antioxidant activity to the oil-water interface by the modified PCs to test the impact on both emulsion physical and oxidative stability. Moreover, the dose-response effect of modified soy and egg PCs on the physical and oxidative stabilities of the emulsions was studied, including the evaluation of lipid oxidation propagation in these heterogeneous systems.

2. Materials and Methods

2.1. Materials

Cod liver oil was provided by Vesteraalens A/S (Sortland, Norway) and kept at −40 °C until use. The peroxide value of the oil was 0.16 ± 0.02 meq. O_2/kg oil. The fatty acid content (%, w/w) of the oil was determined in our lab and reported in a previous study (Yesiltas et al., 2019) as follows: C14:0 (4.0), C16:0 (9.2), C16 (9.2), C16:1n-7 (8.3), C18:0 (2.2), C18:1n-9 (15.8), C18:1n-7 (4.1), C18:2n-6 (2.5), C18:3n-1 (0.2), C20:1n-9 (11.4), C20:5n-3 (8.8), C22:1n-11 (5.4), and C22:6n-3 (11.4). Similarly, α-, β-, γ-, and δ-tocopherol contents were 146 ± 7, 0 ± 0, 97 ± 2, and 43 ± 0.3 μg toc/g oil, respectively [18]. Sodium caseinate, CAS, was provided by Arla Foods Ingredients amba (Viby J, Denmark) with 92% protein content. Commercial phosphatidylcholine (CPC) (Lipoid S 100, phosphatidylcholine from soybean) was provided by Lipoid GmbH (Ludwigshafen am Rhein, Germany). Modified soy and egg phosphatidylcholines with covalently attached caffeic acid (SPC and EPC,

respectively) were synthesized as described previously [22]. Briefly, soy and egg lecithin were enzymatically hydrolyzed in 90% v/v ethanol-water solutions using Lipozyme RM IM from Novozymes. The respective lysophosphatidylcholines were purified from the mixtures, followed by coupling to caffeic acid (Figure 1) [22] to generate the modified PCs (SPC and EPC). The modified PCs were purified from the reaction mixtures using column chromatography, flushed under nitrogen, and frozen until further characterization and structural elucidation [22].

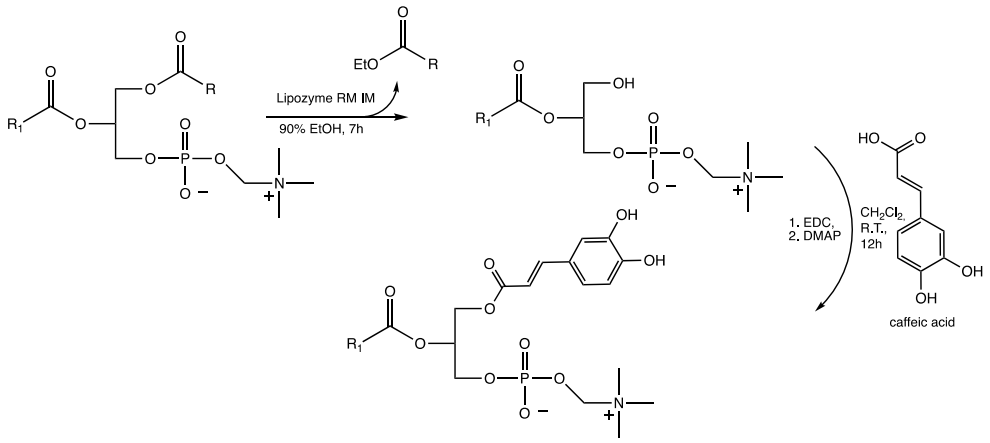


Figure 1. Synthetic route for the synthesis of Sn-1-caffeoyl and Sn-2-acyl phosphatidylcholine, starting with egg/soy PC.

The fatty acid content (%, w/w) of the CPC was determined as follows: 16:0 (13.42), C18:0 (3.7), C18:1n-9 (10.97), C18:1n-7 (1.22), C18:2n-6 (62.61), C18:3n-3 (6.96), SPC was determined as follows: 16:0 (3.15), C18:0 (1.13), C18:1n-9 (13.24), C18:1n-7 (0.78), C18:2n-6 (74.66), C18:3n-3 (6.78), and EPC were determined as follows: 16:0 (3.79), C18:0 (2.09), C18:1n-9 (51.68), C18:1n-7 (1.45), C18:2n-6 (23.56), C18:3n-3 (0.10), C20:4n-6 (8.31), C22:6n-3 (1.99). All solvents and chemicals used were of analytical grade.

2.2. Methods

2.2.1. Emulsion Production

Emulsions were produced as described previously [18], where aqueous phases were prepared by dissolving CAS, CPC, SPC, and EPC in distilled water and stirring overnight at 4 °C (Table 1). Before the emulsification process, the pH of the aqueous phases was adjusted to 7 using 2 M NaOH. Emulsions were produced in 500 g batches using a Stephan Universal mixer (Stephan, UMC5, 1995, Hameln, Germany) by starting with stirring the aqueous phase and slowly adding the oil into the aqueous phase. Ferrous (Fe^{2+}, 100 µM) and sodium azide (0.05% w/v) were added to emulsions to accelerate lipid oxidation and prevent microbial growth, respectively. After production, emulsions were divided into 100 mL bottles of approximately 90 g and stored for 12 days at room temperature in darkness. Physical and oxidative stability parameters were assessed throughout the storage.

Table 1. Emulsifier and caffeic acid content of emulsion codes. All emulsions contain 70 wt.% fish oil, 2.8 wt.% total emulsifier, and 1.2 as the ratio of CAS to total PC.

Emulsion Code [1]	Percentage of the EPC/SPC of Total PC	Theoretical Caffeic Acid Concentration in ppm [2]
1-CAS	-	-
2-CPC	-	-
3-SPC_L	10%	360
4-SPC_M	30%	1080
5-SPC_H	60%	2160

Table 1. *Cont.*

Emulsion Code [1]	Percentage of the EPC/SPC of Total PC	Theoretical Caffeic Acid Concentration in ppm [2]
6-EPC_L	10%	360
7-EPC_M	30%	1080
8-EPC_H	60%	2160
9-CPC_L	-	360
10-CPC_H	-	2160

[1] CAS: sodium caseinate; CPC: commercial phosphatidylcholine; EPC: modified egg phosphatidylcholine; SPC: modified soy phosphatidylcholine; L: low concentration of caffeic acid; M: medium concentration of caffeic acid; H: high concentration of caffeic acid. [2] The caffeic acid concentrations are calculated in ppm based on the ratio between com. PCs and mod. PCs. Due to experimental challenges, the ranges for final caffeic acid concentrations were 349, 1041, and 2082 ppm for SPC and 344, 1025, and 2051 ppm for EPC.

2.2.2. Physical Stability of Emulsions

Creaming Index

Creaming was measured throughout the storage on days 1, 2, 5, 8, and 12 (Table S1). Table 2 shows the results obtained on days 1 and 12. The creaming index was calculated based on the equation below:

$$\text{Creaming index (\%)} = (b/a) \times 100$$

where a is the total height of the emulsion in the tube, and b is the height of the clear aqueous phase at the bottom of the tube.

Droplet Size

The droplet size of the emulsions was determined using the laser diffraction technique in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK). Samples were collected for measurement on days 0 and 12 according to the method described previously [17]. The volume-weighted (D[4,3]) mean diameter was calculated based on the equation below:

$$D[4,3] = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$$

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 duplicate.

Zeta Potential

The surface charge of the emulsion droplets was determined using Zetasizer Nano 2S (Malvern Instruments, Ltd., Malvern, UK). Samples were prepared by diluting 0.32 g of emulsion in 40 g of distilled water, followed by mixing using a vortex. DTS-1070 disposable capillary cells (Malvern Instruments, Ltd., Malvern, UK) were used for loading the samples. The zeta potential range was set to −100 to +50 mV, and measurements were performed at 25 °C. The measurements were performed on day 2 in duplicate.

Apparent Viscosity

The apparent viscosity of the samples was measured using a stress-controlled rheometer equipped with a standard bob cup system (CC25) (Stresstech, Reologica Instruments AB, Lund, Sweden). Emulsions (15 g per replicate) were measured over a shear stress range of 0.0125–50 Pa at 25 °C. Results were calculated based on a specific shear rate of 20 s^{-1} for each emulsion in Pascal seconds (Pa·s). Samples were measured on days 1 and 12 in duplicate.

2.2.3. Oxidative Stability of Emulsions

Primary Oxidation Products—Peroxide Value

Primary oxidation products were reported using peroxide value (PV), which is a Fe^{2+} oxidation-based spectrophotometric method. Lipids were separated using the Bligh and Dyer method with slight changes [23]. Five g of emulsion was used for extracting lipids with a reduced amount of solvent (10 mL of methanol and chloroform, 1:1). PV was measured on the lipid extracts by colorimetric determination of iron thiocyanate on a spectrophotometer (Shimadzu, UV mini 1240, Kyoto, Japan) at 500 nm [24] in duplicates.

Tocopherol Content

Tocopherols were determined using HPLC (Agilent 1100 Series; Column: Waters Spherisorb 3 µm Silica; 4.6 × 150 mm). Analysis was performed according to the AOCS Official Method Ce 8-89 [25] using the same lipid extracts obtained previously (Section Primary Oxidation Products—Peroxide Value), which were further evaporated and dissolved in heptane. Measurements were performed in duplicate.

Secondary Oxidation Products—Volatile Compounds

Volatile secondary oxidation products were analyzed according to the method described by [26]. Volatile compounds were trapped on Tenax GR tubes using dynamic headspace followed by GC-MS. The volatile compounds were separated in a gas chromatograph (Agilent Technologies, 6890N Network GC System, Wilmington, 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, Santa Clara, 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) were used for the identification. 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 identified and analyzed in all emulsion samples.

Fluorescence Microscopy

Details of the approach used and the associated instrumentation have been published [27,28]. Briefly, C11-BODIPY$^{581/591}$ was used as the fluorescent probe to monitor the extent of oxidation. This molecule localizes at the oil-water interface, with an oxidation-dependent change in the color of fluorescence from red (unoxidized) to green (oxidized). For these experiments, oxidation was initiated by exposing the surface of the oil droplet to a localized population of singlet molecular oxygen, $O_2(a^1\Delta_g)$, created by the two-photon excitation of a hydrophilic $O_2(a^1\Delta_g)$ photosensitizer using a tightly focused laser beam. A key feature of this approach is that products of the initial localized photoinitiated reaction of $O_2(a^1\Delta_g)$ with the lipid (e.g., alkoxyl and peroxyl radicals) can propagate over the surface and through the oil droplet to oxidize distant C11-BODIPY$^{581/591}$ molecules, a process revealed upon recording fluorescent images at elapsed times after $O_2(a^1\Delta_g)$ production.

2.2.4. Statistical Analysis

Statgraphics XVII (Statpoint Technologies, Inc., Warrenton, VA, 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 of $1 - \alpha = 95\%$.

3. Results and Discussion

3.1. Physical Stability of Emulsions

All the produced emulsions presented creaming lower than 14% during 12-day storage (Table 2), which denotes that the emulsifier evaluated provided high physical stability. In fact, we did not observe a significant increase in droplet size (D4,3) during storage for any emulsions, except for the emulsion code 7-EPC-M, which slightly increased its D4,3 value

from 12.67 ± 0.02 μm at day 0 to 12.96 ± 0.01 μm at day 12. Interestingly, the replacement of caseinate with non-modified PC resulted in emulsions with significantly higher D4,3 values when compared to those stabilized with only caseinate (Table 2). Moreover, increasing the concentration of modified PC significantly reduced the droplet size of the emulsions in a similar manner for PCs from both sources (soybean and egg) (Table 2). These findings denote a superior emulsifying activity of modified PCs when compared to the commercial PC, which was attributed to a higher HLB value for the former (i.e., a larger polar group after antioxidant addition) that leads to better dispersion in the aqueous phase [18]. The good emulsifying properties of the modified PCs could be due to their different structure, where a small molecule (caffeic acid) has replaced a long fatty acid, thereby resembling a lyso-PC, which is known to have better emulsifying properties than the commercial PC with two fatty acid chains. Moreover, the conformation of the modified PCs can be affected by the addition of a benzene ring due to the attached caffeic acid having a less hydrophobic group in contrast to the alkyl chain in the surfactant tail. Our results indicated that the differences in fatty acid composition of the modified soybean or egg PCs did not significantly affect their emulsifying activity. In any case, it should be noted that 75–80% of the fatty acids present in both soybean and egg PC had a chain length of 18 carbon atoms, ruling out the effect of chain length on the adsorption potential of the modified emulsifier [18].

All emulsions present a markedly negative zeta potential (<−40 mV) since they were produced at pH 7, which is above the isoelectric point of casein (Table 2). In addition, although PC is zwitterionic, it has also been reported to contribute to the negative charge under alkaline conditions [22]. We did not find significant differences in the zeta potential of the emulsions, except for the emulsion stabilized with casein and commercial PC that had a slightly less negative value (Table 2). These results are in line with our previous study evaluating the use of modified soybean PC with different chain lengths for the stabilization of high-fat fish oil-in-water emulsions [18]. It is worth mentioning that the combination of emulsifiers used provided sufficient electrostatic repulsions between droplets, avoiding flocculation and coalescence phenomena and conferring physical stabilization of the emulsions. Moreover, the physical stability of the oil-water interface and thereby the oil droplets can also be affected by the formed oxidation products. Nonetheless, the negatively charged interfacial film is notoriously detrimental to the oxidative stability of emulsions when lipid oxidation is catalyzed by metal ions present in the aqueous phase [29].

Emulsions showed shear-thinning behavior, with apparent viscosity at 20 s^{-1} ranging from 0.3 to 2.7 Pa·s (Table 2). As previously reported for both low-fat [14] and high-fat emulsions [18], the partial replacement of caseinate in the formulation by either commercial or modified PCs resulted in a significant decrease in the apparent viscosity. This is explained by the significantly smaller droplet size of the emulsion stabilized with only caseinate, which leads to more friction between oil droplets as the surface-to-volume ratio of the oil phase increases [18,30]. On the contrary, the decrease in droplet size found when increasing the content of modified PCs did not correlate with the observed decrease in apparent viscosity (Table 2). Therefore, other factors, such as casein content in the aqueous phase, which has also been attributed to influencing viscosity, might be responsible for the values obtained [18]. It is worth mentioning that, except for the emulsion stabilized with only caseinate, emulsions decreased their apparent viscosity during storage. Similarly, a decrease in the apparent viscosity of high-fat emulsion systems such as mayonnaise during storage has been reported in the literature, which was attributed to changes in droplet size [31].

Table 2. Creaming index, volume-weighed diameter (D[4,3]), apparent viscosity, and zeta potential of the high-fat emulsions stabilized by sodium caseinate (CAS) and/or commercial/modified phosphatidylcholines (PC). All emulsions contain 70 wt.% fish oil, 2.8 wt.% total emulsifier, and 1.2 as the ratio of CAS to total PC. Emulsion 1-CAS contains only 2.8% sodium caseinate.

Emulsion Code	Creaming Index (%)		D[4,3] (μm)		Zeta Potential (mV)	Apparent Viscosity (Pa·s) at 20 s^{-1}	
	Day 1	Day 12	Day 1	Day 12	Day 2	Day 1	Day 12
1-CAS	0	0	8.56 ± 0.00^{a}	7.54 ± 0.45^{a}	-49.8 ± 2.9^{a}	2.66 ± 0.13^{e}	$2.97 \pm 0.05^{h,*}$
2-CPC	0	2	17.93 ± 0.09^{e}	17.93 ± 0.73^{e}	-41.2 ± 4.0^{b}	0.94 ± 0.01^{d}	0.75 ± 0.01^{f}
3-SPC_L	0	2	15.81 ± 0.03^{d}	15.31 ± 0.01^{d}	-48.3 ± 2.3^{a}	0.90 ± 0.03^{d}	0.83 ± 0.02^{g}
4-SPC_M	0	5	13.32 ± 0.18^{c}	13.04 ± 0.68^{c}	-48.3 ± 1.6^{a}	0.76 ± 0.01^{c}	0.61 ± 0.00^{d}
5-SPC_H	1	7	11.91 ± 0.02^{b}	11.32 ± 0.28^{b}	-46.4 ± 3.7^{ab}	0.53 ± 0.03^{b}	0.40 ± 0.00^{b}
6-EPC_L	0	2	16.48 ± 0.24^{d}	16.55 ± 0.01^{d}	-46.7 ± 2.2^{ab}	0.89 ± 0.02^{d}	0.84 ± 0.00^{g}
7-EPC_M	0	3	12.67 ± 0.02^{bc}	$12.96 \pm 0.01^{c,*}$	-49.8 ± 1.4^{a}	0.87 ± 0.02^{d}	0.71 ± 0.00^{e}
8-EPC_H	0	4	11.86 ± 0.20^{b}	11.59 ± 0.50^{b}	-49.5 ± 5.0^{a}	0.79 ± 0.03^{c}	0.54 ± 0.00^{c}
9-CPC_L	2	6	17.02 ± 0.39^{de}	16.48 ± 1.15^{d}	-46.5 ± 3.4^{ab}	0.76 ± 0.01^{c}	0.76 ± 0.02^{f}
10-CPC_H	6	14	22.83 ± 2.31^{f}	$27.77 \pm 1.79^{f,*}$	-46.8 ± 5.4^{ab}	0.34 ± 0.01^{a}	0.23 ± 0.00^{a}

Letters a–h denote significant differences between samples at a certain sampling point ($p < 0.05$). An asterisk (*) denotes a significant increase during storage.

3.2. Oxidative Stability of Emulsions

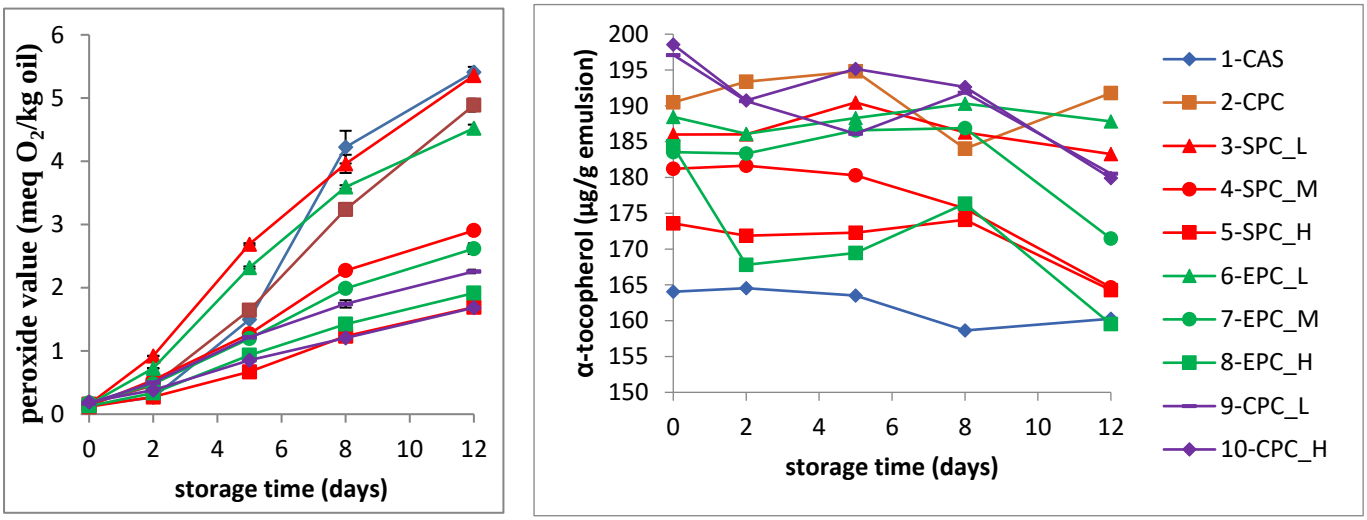
3.2.1. Formation of Primary Oxidation Products

Lipid hydroperoxide formation in the emulsions was reported during 12 days of storage (Figure 2a). The emulsion produced with only CAS had 2 days of lag phase, whereas the emulsions produced with both CAS and PCs had a significant increase in their peroxide value already after day 0 and kept increasing throughout the storage (Supplementary Material, Table S2). However, on day 12, all emulsions substituted with PCs were more or equally oxidatively stable compared to emulsions produced with only CAS, with a PV of 5.41 ± 0.08 meq. O_2/kg oil (Table S2). All the emulsions with modified PCs were more oxidatively stable compared to emulsions with only CAS or CAS and commercial PC, except for the emulsion with modified soy PC at its low concentration on day 12 (see 3-SPC_L in Figure 2a and Table S2). Free caffeic acid (CA) emulsions (9-CPC-L and 10-CPC_H) were more or equally oxidatively stable at their equivalent CA levels (Figure 2a, Table S2). On the last day of storage (day 12), soy PC was slightly better compared to egg PC when the CA level was 2160 ppm, whereas the opposite trend was observed for 360 and 1080 ppm CA levels. Results obtained in this study are comparable to a previous study where emulsions produced with CAS and PCs that are modified with caffeic acid and chain lengths at C14 and C16 and used in similar concentrations as in this study (360, 1080, and 2160 ppm as the final concentration of caffeic acid) ranged between ~2 and 6 meq. O_2/kg oil [18]. Similarly, in another study where 70% fish oil-in-water emulsions were stabilized with CAS and DATEM, the highest PV observed was 4.7 meq. O_2/kg oil for the control emulsions without any caffeic acid [17]. On the other hand, high-fat emulsions stabilized with CAS and dodecyl succinylated alginate, or CAS, and commercial alginate showed lower oxidative stability (8.0 ± 0.8 and 9.0 ± 1.4 O_2/kg oil, respectively) [26].

3.2.2. Consumption of Tocopherols

Tocopherols naturally exist in cod liver oil and are used as antioxidants as oxidation develops during storage. A-, γ-, and δ-tocopherols were identified in these emulsions and reported in Figure 2b and Supplementary Material Figure S1. Tocopherols were not consumed extensively during storage, which overall indicates good oxidative stability of the emulsions. This shows that the emulsions were not under high oxidative stress, which could lead to a large decrease in the content of tocopherols naturally present in fish oil. As seen in Figure 2b, α-tocopherol content was quite stable during the 12 days of storage. The protection of α-tocopherols could also be attributed to their regeneration in

the presence of PCs via the proton-donating capacity of the amino groups [14,32]. However, we observed some small changes between day 12 and day 0, which might reveal some information regarding the protection obtained by emulsifiers in each emulsion. Overall, α-tocopherols were consumed more in the middle and higher concentrations of the modified PCs as well as in free caffeic acid-added emulsions. The largest decrease between days 0 and 12 was obtained for 8-EPC_H with 13%, followed by 4-PCP_M and 10-CPC_H with 9%. At low concentrations of caffeic acid, there was no or only a 1% decrease for the modified PCs, 6-EPC_L and 3-SPC_L, respectively, whereas commercial PC and caffeic acid (9-CPC_L) decreased 8%. This could be explained by the availability of caffeic acid at the oil-water interface for the modified PCs, where it was used as a radical scavenger instead of α-tocopherol in the oil phase. At higher concentrations of caffeic acid, we did not observe a clear pattern for emulsions with modified PCs versus emulsions with commercial PC and caffeic acid. Similarly, in a previous study, no significant difference was observed in the consumption of α-tocopherol content between emulsions produced with a higher concentration of modified PCs versus emulsions with commercial PCs at the same concentration of added caffeic acid [18].



(**a**) (**b**)

Figure 2. Lipid oxidation in high-fat oil-in-water emulsions during 12 days of storage: (**a**) Formation of hydroperoxides measured using peroxide value; (**b**) Consumption of α-tocopherols detected using HPLC.

3.2.3. Development of Volatile Secondary Oxidation Products

Volatile compounds formed in emulsions during 12-day storage were identified and quantified. Four volatile compounds, 1-penten-3-ol, 2-pentenal, (*E*,*E*)-2,4-heptadienal, and hexanal, were selected based on their high concentrations and their representative trends for the rest of the volatile compounds (Figure 3). The rest of the volatiles were included in the Supplementary Material, Figure S2. Overall, emulsions containing a high content of modified PCs (5-SPC_H and 8-EPC_H) provided better or similar oxidative stability compared to emulsions containing commercial PC and free caffeic acid (10-CPC_H). The development of 1-penten-3-ol indicated that replacing some of the CAS with commercial PC did not contribute significantly to its oxidative stability; however, replacing some of the commercial PC with modified PCs improved oxidative stability significantly independently of the source of PC (Figure 3a). Furthermore, increasing the concentration of modified PCs decreased lipid oxidation, and modified PCs were more effective than the combination of commercial PC and free caffeic acid at the same concentration.

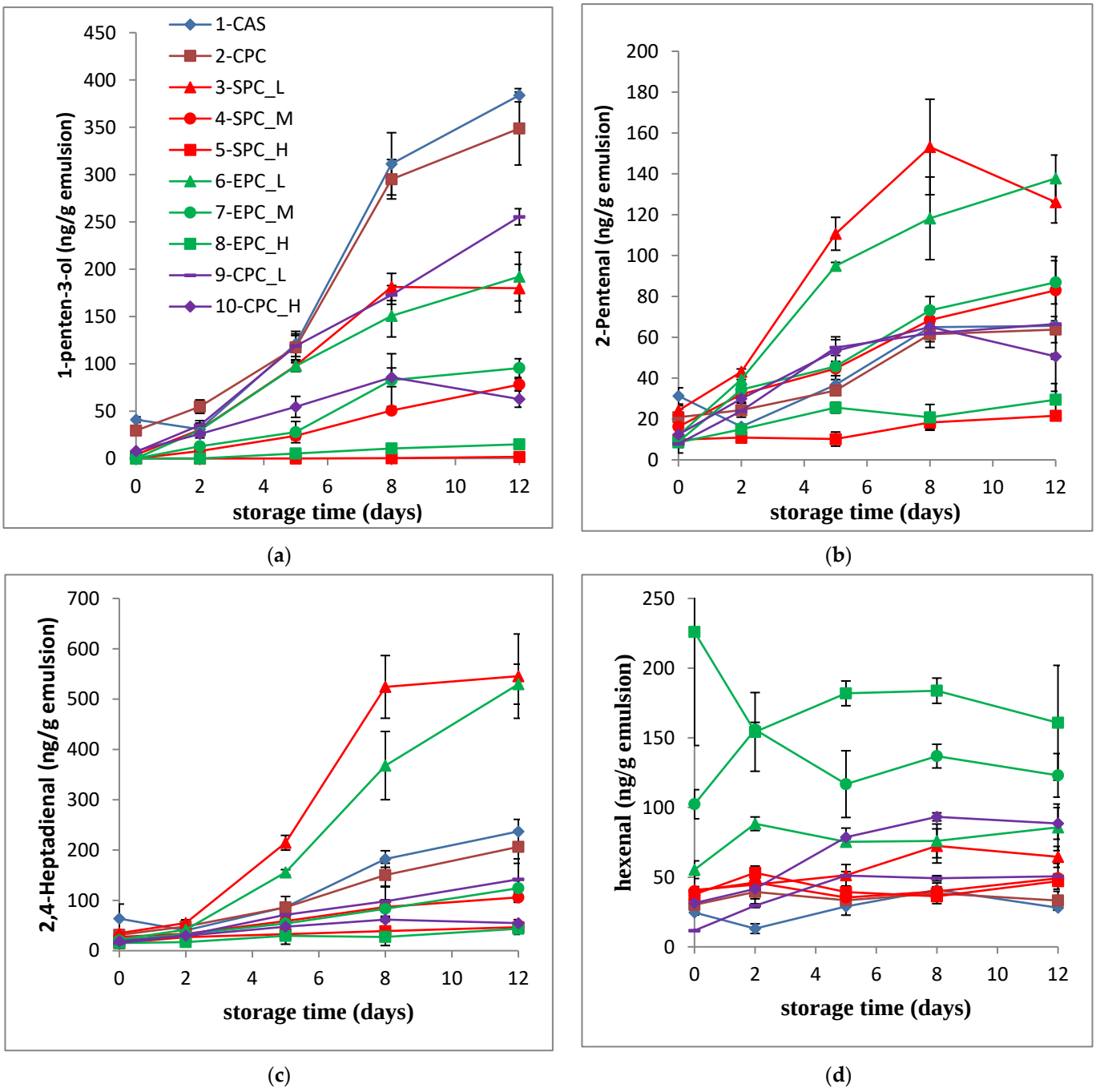


Figure 3. Lipid oxidation in high-fat oil-in-water emulsions during 12 days of storage: Development of secondary oxidation volatile compounds using GC-MS (**a**) 1-penten-3-ol; (**b**) 2-pentenal; (**c**) (*E*,*E*)-2,4-heptadienal; and (**d**) hexanal.

A similar trend was also observed for 2-pentenal and (*E*,*E*)-2,4-heptadienal (Figure 3b,c). However, for these two volatile compounds, a prooxidant effect of modified PCs was found at their low concentrations (3-SPC_L and 6-EPC_L) compared to the emulsions with only CAS or commercial PCs (1-CAS and 2-CPC). Oxidation levels were approximately 2-fold higher for 3-SPC_L and 6_EPC_L. The level of (*E*,*E*)-2,4-heptadienal (around 600 ng/g emulsion) was similar to what was found for 70% fish oil-in-water emulsion stabilized with CAS and modified alginate, where the modification of the alginate resulted in a prooxidative effect due to a double bond in its alkyl chain [26]. On the contrary, this prooxidant effect was not observed for commercial PC with added free caffeic acid at a

low concentration (9-CPC_L), which highlights the effect of bringing caffeic acid to the oil-water interface. The large difference in oxidative stability could be attributed to the fact that the concentration of the antioxidant at the oil-water interface is important for its activity. A similar effect was also observed in a previous study, where emulsions containing modified PCs with different chain lengths (C14 and C16) and covalently attached caffeic acid had a prooxidative effect when used in low concentrations at 360 ppm [18]. On the other hand, the reason behind the prooxidant effect for the two above-mentioned volatiles and not for hydroperoxides or 1-penten-3-ol was presumably due to the 3-SPC_L and 6-EPC_L promoting the decomposition of PV in these two volatiles at the expense of other volatiles. This could be attributed to the well-known phenomenon related to the presence of tocopherols, which was reported previously by Karahadian and Lindsey, that the tocopherols directed the formation of 2,4-heptadienals [33]. Similar effects have been observed in some of our other studies for (*E*,*E*)-2,4-heptadienal [18,19].

Hexanal, which stems from n-6 fatty acids, showed a different trend than the other three volatile compounds presented, which are derived from n-3 fatty acids (Figure 3d). Overall, emulsions produced with only CAS or CAS and commercial PC (1-CAS and 2-CPC) had similar or higher oxidative stability compared to others. Specifically, modified soy PC at medium and high concentrations (4-SPC_M and 5-SPC-H), as well as commercial PC with caffeic acid added at low concentration (9-CPC_L), had similar oxidative stability as 1-CAS and 2-CPC. The most oxidized samples were emulsions containing modified egg PCs at their medium and high concentrations (7-EPC_M and 8-EPC_H), followed by commercial PC emulsions with free caffeic acid at their high concentration (10-CPC_L). At low concentrations of caffeic acid, hexanal formation was the highest in emulsions containing modified soy PC (3-SPC_L) compared to medium and high concentrations (4-SPC_M and 4-SPC_H), thus the prooxidant effect was similar to other volatiles mentioned before. However, this effect was not seen for the modified EPC emulsions, where the oxidation significantly increased with increasing concentrations of modified egg PC and thereby caffeic acid concentration. This is due to the differences in the fatty acid content of the soybean and egg PCs. SPC has a much higher content of 18:2n-6, whereas EPC has a significantly higher level of 20:4n-6. Therefore, it is expected to have higher levels of hexanal in the emulsions containing EPC due to the oxidation of 20:4n-6. Moreover, the hexanal levels at day 0 were already in proportion to the EPC concentration used in the emulsions, as shown in Figure 3d.

According to all the volatiles, in general, modified soy PC performed better compared to modified egg PC in controlling the development of volatile compounds. Low concentrations of modified PCs were generally prooxidants compared to their medium and high concentrations. At high concentrations of PC addition, it was more effective to use modified PCs with covalently attached caffeic acid compared to commercial PCs with an addition of free caffeic acid independent of the food source (eggs vs. soybeans). On the other hand, it is well known that the physical properties (oil-water interface composition, thickness, charge, etc.) of emulsions also affect the development of lipid oxidation [12]. We identified that the emulsions containing modified PCs provided smaller droplets and a slightly more negative surface charge compared to the emulsions with commercial PC, indicating a larger surface area for the former emulsions and a slightly stronger attraction of prooxidant metal ions to the interface. However, the overall performance of the modified PCs in controlling lipid oxidation was not greatly affected by the smaller droplets and thereby larger surface area. Moreover, the impact of bringing caffeic acid to the interface was more effective in providing oxidative stability than the advantages provided by the physical characteristics of emulsions. In the case of maintaining a similar droplet size as in the commercial PC emulsions, the oxidative effect obtained by the modified PCs could be expected to be more pronounced. In order to understand the mechanism of oxidation development and antioxidant activity at the oil-water interface of an emulsion, complementary techniques, such as advanced microscopy, are required.

3.2.4. Propagation of Oxidation Using Fluorescence Imaging

To complement the data reported in the preceding sections, fluorescence imaging experiments were performed on one of the emulsions (6-EPC_L) to monitor the propagation of oxidation initiated in a spatially confined domain at the droplet surface (Figure 4) [27,28]. For these studies, singlet molecular oxygen, $O_2(a^1\Delta_g)$, was used as the agent to initiate oxidation. The time and spatial propagation of oxidation of molecules at or near the droplet surface was monitored using a fluorophore that shows a color change upon interaction with reactive oxygen species commonly formed in lipid oxidation (i.e., hydroxyl, alkoxyl, and peroxyl radicals).

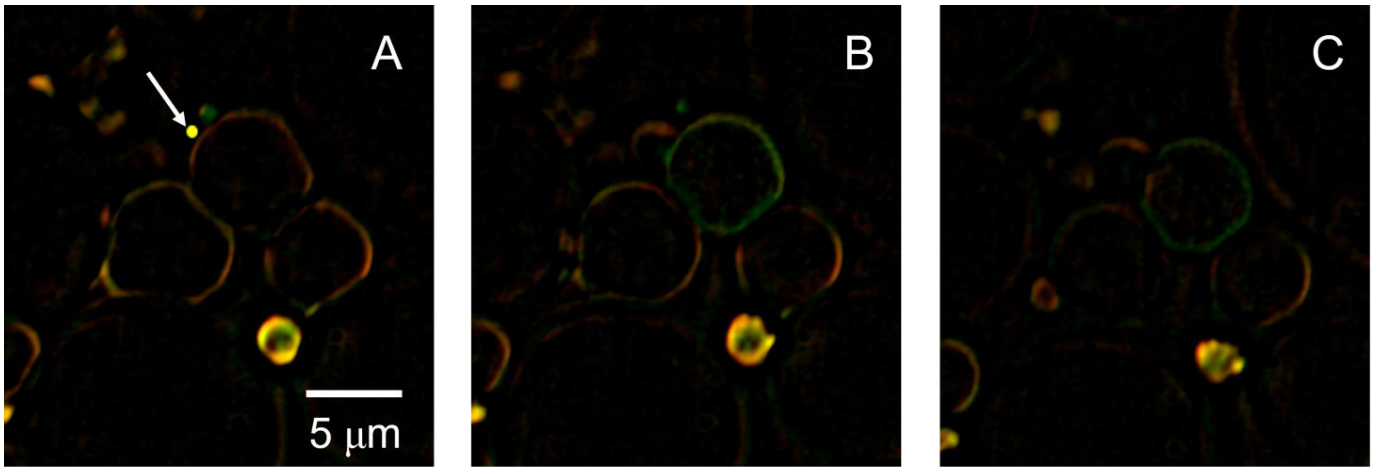


Figure 4. Images of 6-EPC_L droplets based on the fluorescence of the oxidative probe C11-BODIPY$^{581/591}$ that localizes at the droplet surface. The unoxidized probe has a red color, whereas the oxidized probe is green. (**A**) Oxidation was initiated by two-photon excitation of a water-soluble $O_2(a^1\Delta_g)$ sensitizer using focused laser light (a yellow dot pointed with an arrow). (**B**) Image recorded after 10 min of continuous irradiation. (**C**) Image recorded after an additional 1 h without irradiation.

A key observation in these studies is that, over the period of observation, oxidation only occurs on the droplet immediately adjacent to the localized site of $O_2(a^1\Delta_g)$ production; oxidation does not readily propagate across the water barrier separating one oil droplet from another. We find this to be a general phenomenon in such systems [27,28]. At the very least, these data provide insight into what added stabilizers are most effective in mitigating oxidation in such emulsions.

In an early study by other investigators on radical-initiated oxidation reactions in 30% oil-in-water emulsions stabilized with Tween 20, the data obtained were interpreted to indicate that oxidation could indeed propagate from one oil droplet to another [34]. However, the same authors reversed themselves in a later article using spatially localized radical-initiated oxidations in the same oil-in-water emulsion, reporting that there were, in fact, no indications of the oxidation propagating from one droplet to another [35]. As such, our present results are consistent with those of others.

The spatial propagation of lipid autoxidation has been discussed in a recent review [36]. In this review, the authors describe three mechanisms for the transfer of oxidants and antioxidants and discuss the transfer speed for the suggested mechanisms as well as the effect of the location and hydrophobicity/amphiphilicity of the molecules.

4. Conclusions

High-fat emulsions produced with sodium caseinate and commercial and modified PCs had increased creaming instability and decreased viscosity compared to emulsions produced with only sodium caseinate. Droplet size decreased with increasing concentration of modified egg/soy PC with covalently attached caffeic acid when compared to non-modified PC. Modified egg/soy PCs provided more negative zeta potential compared

to commercial PCs. The development of lipid oxidation was the lowest or similar for emulsions containing high concentrations of modified PCs with covalently attached caffeic acid compared to emulsions with commercial PC and free caffeic acid at similar concentrations. Furthermore, the concentration of modified PCs played a crucial role in their prooxidant or antioxidant effects. At low concentrations, some modified PCs showed a prooxidant effect for certain volatile compounds. However, at medium and high concentrations, modified PCs performed better in controlling oxidation than CAS or commercial PCs. Furthermore, the type of PC source influenced oxidative stability, with modified soy PC generally performing better than modified egg PC. The fatty acid composition of the PCs played a role in the formation of specific oxidation products, such as hexanal. Overall, the study demonstrated that modified PCs, especially when combined with CA, can enhance the oxidative stability of high-fat emulsions. The presence of covalently attached antioxidants at the oil-water interface proved to be more effective than the physical characteristics of the emulsions in controlling lipid oxidation. Further research using complementary techniques such as microscopy is essential to understanding the underlying mechanisms and the role of antioxidants in emulsion stability and oxidation control. This study demonstrated that oxidation did not propagate from one oil droplet to another when oxidation was initiated by a spatially confined population of singlet oxygen localized near one oil droplet.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/colloids7030060/s1. Figure S1: (A) Gamma- and (B) delta-tocopherol consumption during 12-day storage. Figure S2: Development of the rest of the volatile compounds (A) 2-ethyl-furan, (B) 1-penten-3-on, (C) 2-hexenal, (D) 4-heptenal, (E) 2-pentyl-furan, (F) 2-heptenal, (G) benzaldehyde, and (H) 2,6-nonadienal during 12-day storage. Table S1: Development of the creaming index during storage. Table S2: Statistical analysis for (a) peroxide value, (b) a-tocopherol, (c) 1-penten-3-ol, (d) 2-pentenal, (e) (*E*,*E*)-2,4-heptadienal, and (f) hexanal.

Author Contributions: Conceptualization, B.Y., P.J.G.-M., A.-D.M.S., Z.G., P.R.O. and C.J.; methodology, B.Y., P.J.G.-M., S.A. and C.B.; validation, B.Y., P.J.G.-M. and C.B.; formal analysis, B.Y. and C.B.; investigation, B.Y., P.J.G.-M., S.A., C.B. and P.R.O.; writing—original draft preparation, B.Y. and P.J.G.-M.; writing—review and editing, B.Y., P.J.G.-M., A.-D.M.S., S.A., Z.G., P.R.O. and C.J.; visualization, B.Y.; supervision, P.J.G.-M., A.-D.M.S., Z.G., P.R.O. and C.J.; project administration, Z.G., P.R.O. and C.J.; funding acquisition, C.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Danish Council for Independent Research Technology and Production Sciences for financing the project Mapping and Characterizing of Lipid Oxidation in Emulsified Systems (MAPOX), grant number DFF–4184-0123A.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We would like to thank Lipoid GmbH, Arla Foods Ingredients amba, and Maritex A/S Norway for donating phosphatidylcholine, sodium caseinate, and cod liver fish oil, respectively. Furthermore, we are grateful to Lis Berner for her skillful work in the laboratory.

Conflicts of Interest: The authors declare no conflict of interest.

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