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ANTIOXIDATIVE EFFECT OF LIPOPHILIZED CAFFEIC ACID IN FISH OIL ENRICHED MAYONNAISE AND MILK

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· Promising antioxidant effectiveness of lipophilized caffeic acid in oil-in water emulsions

· Different *"critical chain length"* for the caffeates in different emulsion systems

· Not predictable *"critical chain length"* of phenolipids in oil-in-water emulsions

· First confirmation of cut-off effect theory in real food systems

Abstract

The antioxidative effect of lipophilized caffeic acid was assessed in two different fish oil enriched food products: mayonnaise and milk. In both emulsion systems, caffeic acid esterified with fatty alcohols of different chain lengths (C1-C20) were better antioxidants than the original phenolic compound. The optimal chain length with respect to protection against oxidation was, however, different for the two food systems. Fish oil enriched mayonnaise with caffeates of medium alkyl chain length (butyl, octyl and dodecyl) added resulted in a better oxidative stability than caffeates with shorter (methyl) or longer (octadecyl) alkyl chains. Whereas in fish oil enriched milk emulsions the most effective caffeates were those with shorter alkyl chains (methyl and butyl) rather than the ones with medium and long chains (octyl, dodecyl, hexadecyl and eicosyl).

These results demonstrate that there might be an optimum alkyl chain length for each phenolipid in each type of emulsion systems.

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1. Introduction

In the last years, several studies have aimed at enriching food products with n-3 polyunsaturated fatty acids (PUFA) of marine origin (Jacobsen, Let, Nielsen, & Meyer, 2008) due to the low intake of n-3 PUFA's in the industrialized world and their known nutritional benefits. However, unsaturated n-3 PUFA's are highly susceptible to oxidation, leading to the development of unhealthy free radicals, reactive aldehydes, and off-flavours with a consequent decrease in the shelf life of the enriched product (Jacobsen et al., 2008; Let, Jacobsen, Sørensen, & Meyer, 2007). In order to tackle this problem different strategies such as antioxidant addition are necessary.

Food products are complex systems containing different phases and constituents (air, water, lipids, proteins, etc). Heterophasic food systems, such as milk and mayonnaise, are widely consumed. Milk and mayonnaise are oil-in-water (o/w) emulsions, which are composed of three phases: oil phase, water phase and an interface between the oil and water phases. The effectiveness of an antioxidant is highly influenced by its interactions with other components (i.e. emulsifier) and its ability to be located in the environment where lipid oxidation takes place. This is known to be at the interface i.e. between the oil and water phases (Coupland & McClements, 1996). In this regard , the so-called polar paradox theory states that lipophilic antioxidants are more effective in oil-in-water emulsions than hydrophilic antioxidants, whereas hydrophilic antioxidants are more effective in oils (Porter, 1993). Based on this theory, phenolic compounds such as caffeic acid, should work better in bulk oils than in emulsions. The lipophilization of phenolic compounds with different alkyl chain lengths will reduce their polarity and thus change their distribution between the different phases in the emulsion. Hence, lipophilization is expected to improve the antioxidant efficacy of polar phenolic compounds.

However, recently several publications have shown that the polar paradox does not accurately predict the behavior of antioxidants and therefore the polar paradox hypothesis needs to be revisited (Laguerre et al., 2009, 2010; Panya et al., 2012; Sørensen et al., 2012). Laguerre et al. (2009) evaluated the antioxidant capacity of different chlorogenate esters in a stripped tung o/w emulsion stabilized with Brij 35 (CAT assay). A non-linear tendency of the antioxidant capacity was observed. These authors reported an increased antioxidative effect with increasing alkyl chain length up to 12 carbon atoms, whereas further increments in the alkyl chain length led to a collapse in the antioxidant effectiveness. This observation was termed "the cut-

off effect" by these authors. The same non-linear tendency was observed with lipophilized rosemarinates; however, for this lipophilized phenolic the maximal antioxidant capacity was obtained with octyl rosmarinate (Laguerre et al., 2010). Later on, Panya et al. (2012) studied the antioxidant efficiency of a homologous series of rosmarinate alkyl esters (C4, C8, C12, C18 and C20) in Tween 20-stabilized stripped soybean o/w emulsion in a storage experiment. In their study, the rosmarinates with shorter fatty acyl chains (C4, C8 and C12) were much better antioxidants than rosmarinic acid and its octadecyl (C18) and eicosyl (C20) esters.

It is important to note, that several studies have been published assessing different esterified phenolic compounds in simple oil-in-water emulsions. However less attention has been paid to their effectiveness in real food matrices. Sørensen et al. (2012) studied the effect of lipophilized rutin and dihydrocaffeic acid in fish oil enriched milk by comparing the native phenolic compounds with medium and long alkyl chain esters. The lipophilized esters evaluated were rutin laurate, rutin palmitate, octyl dihydrocaffeate and oleyl dihydrocaffeate. It was concluded that for both types of compounds, the medium chain esters were better antioxidants than the long chain esters and the non-lipophilized phenolics. Besides, they pointed out the necessity of further studies in order to understand the antioxidant capacity of the lipophilized compounds regarding their chain length esterified to the phenolic compound and the type of food emulsion system.

Therefore, the aim of the present study was to evaluate the antioxidant effect of caffeic acid and its esters (caffeates) in fish oil enriched mayonnaise and milk emulsions. In mayonnaise caffeic acid and caffeates C1-C18 and in milk caffeic acid and caffeates C1-C20 were evaluated as antioxidants during storage.

2. Material and Methods

2.1. Materials

Rapeseed oil and fish oil were supplied by Maritex A/S a subsidiary of TINE, BA (Sortland, Norway). Rapeseed oil, used in the mayonnaise preparation, had a peroxide value (PV) of 0.3 meq peroxides/kg oil and a tocopherol content of 205 mg α-tocopherol/kg, 68 mg β-tocopherol/kg and 292 mg γ-tocopherol/kg. Finally, the fatty acid composition was as follows: 16:0, 4.5%; 18:0, 1.5%; 18:1n-9, 57.2%; 18:1n-7, 2.5%; 18:2n-6, 20.1% and 18:3n-3, 10.2%.

The fish oil, used in both milk and mayonnaise productions, had a PV of 0.3 meq peroxides/kg oil and tocopherol content of 249 mg α-tocopherol/kg, 98 mg γ-tocopherol/kg and 47 mg δ-tocopherol/kg. The fatty

86 acid composition was as follows: 14:0, 3.5%; 16:0, 9.9%; 16:1n-7, 8.8%; 18:0, 2.0%; 18:1n-9, 16.3%; 18:1n-
87 7, 4.9%; 18:2n-6, 1,8%, 18:3n-3, 2.6%, 18:4n-3, 2.6%, 20:1n-7, 12.6%; 20:5n-3 (EPA), 9.16%; 22:1n-9,
88 5.8%, 22:5n-3, 1.1% and 22:6n-3 (DHA) 11.1%. The total percentages of n-3 and n-6 PUFA in this oil were
89 24.0 and 1.8 %, respectively.

90 Potassium sorbate used was purchased from Merck (Dramstadt, Germany). Grindsted FF DC stabilizer (guar
91 gum and sodium alginate) was donated by Dupont, Danisco Ingredients (Brabrand, Denmark). Fresh milk
92 (0.5 and 1.5% fat content), salt (sodium chloride), sugar, lemon juice, estragon vinegar and egg yolk were
93 purchased in a local market.

94 Caffeates were synthesized in an acid catalyzed reaction (sulfuric acid) with caffeic acid and fatty alcohols as
95 described elsewhere (Sørensen et al., 2014). Caffeic acid and fatty alcohols were purchased from Sigma
96 Aldrich (Steinheim, Germany).

97 All other chemicals used were of HPLC grade and purchased from Lab-scan (Dublin, Ireland). The external
98 standards used for the identification and quantification of the secondary oxidation compounds were from
99 Sigma Aldrich..

100 **2.2. Experimental design and production of mayonnaise and milk**

101 Fish oil enriched mayonnaise and milk were produced according to the experimental design in Table 1.
102 Caffeic acid and lipophilized derivatives of caffeic acid (caffeates) were assessed as antioxidants in fish oil
103 enriched mayonnaise and milk. In the mayonnaise experiment, the different caffeates selected were: methyl,
104 butyl, octyl, dodecyl and octadecyl caffeates, and in the milk experiment, the selected caffeates were:
105 methyl, butyl, octyl, dodecyl, hexadecyl and eicosyl caffeates.

106 All antioxidants were tested at 100 µM. To evaluate the effect of antioxidant concentration in mayonnaise,
107 one additional treatment was included; octyl caffeate added at 200 µM. Octyl caffeate was selected based on
108 earlier results in o/w emulsion (CAT assay), where this ester was most efficient (Sørensen et al., 2013).

109 Mayonnaise batches of 500 g were prepared under vacuum using a Stephan Universal mixer (Stephan
110 UMC5, Hameln, Germany). The production of mayonnaises at these conditions assures physical stable
111 emulsions as has been probed in previous studies (Jacobsen, Adler-Nissen, & Meyer, 1999; Let et al.,

2007). Each batch contained by weight 64% rapeseed oil, 16% fish oil, 9.25% water, 4% estragon vinegar, 4% egg yolk, 1.2% lemon juice, 1.0% sugar, 0.3% salt (sodium chloride), 0.15% Grindsted FF DC and 0.1% potassium sorbate. All antioxidants were dissolved in 1 mL methanol and thereafter added in the water phase before mayonnaise production to give a final concentration of 100 µM and for mayonnaise with octyl caffeate also 200 µM. In the mayonnaise without antioxidant (Mayo_CONTROL), 1 mL methanol was added.

Mayonnaises were stored in 100 mL brown bottles, at 20ºC for 4 weeks in darkness. Samples were taken at day 0, 3, 6, 9, 12, 15, 21 and 28 and subdivided into 50 mL brown bottles, flushed with N_2 and stored at -40ºC until analyses.

Milks with 0.5 and 1.5% fat were mixed (1:1, w/w) to obtain a total fat content of 1%. Subsequently, the milk was heated to 72ºC for 15 s and the fish oil (0.5%, w/w) and the antioxidant were added. This mixture was then homogenized using a two valve table homogenizer from GEA Niro Soavi Spa (Parma, Italy). The pressure was set at 250 bar with four circulations of the emulsion. Using these conditions, stable milk emulsions are achieved as has been proven in previous studies (Sørensen et al., 2007, 2012). Similarly to the mayonnaise, all antioxidants were dissolved in methanol (1.95 mL) and was subsequently added to milk to give a finally antioxidant concentration of 100 µM. The same volume of methanol was added to the milk without antioxidant (Milk_CONTROL).

Milk emulsions were stored in 100 mL sterilized bottles at 5ºC. Samples were taken at day 0, 3, 6, 9 and 12 and subdivided into 50 mL brown bottles, flushed with N_2 and stored at -40ºC until analyses.

Storage temperatures and times for mayonnaise and milk were selected according to previous mayonnaise and milk experiments. The analyses performed at each storage time were peroxides, volatile compounds and tocopherol content. The fatty acid composition was assessed at the beginning and end of the storage period (day 0 and 28 for mayonnaise, and day 0 and 12 for milk).

2.3. Extraction of lipids from mayonnaise and milk

Peroxide value (PV), fatty acid composition (FAME) and tocopherol concentrations were measured on the lipids of the sample. Thus, prior to these analyses, lipids were extracted from mayonnaise and milk

emulsions. Frozen mayonnaise samples were thawed and centrifuged (2500g, 10 min, 4ºC). The oil (upper phase) was then separated and used for the different analyses.

Lipids were extracted from fish oil enriched milk according to the method described by Iverson, Lang, & Cooper (2001) based on the method of Bligh and Dyer (1959). For each sample two lipid extractions were performed.

2.4. Fatty acid composition (FAME)

The lipid extract obtained from the milk was evaporated under nitrogen. Thereafter, the glycerol bound fatty acids were first transesterified with methanolic NaOH (0.5 M). Then hydrolytically released and free fatty acids were methylated by a boron trifluoride reagent (20%) catalyzed process (AOCS, 1998) Oil phase from mayonnaise were weighted in vials and methylated using a slightly modified version of the above procedure in which the methylation was carried out in one step using a microwave (Multiwave3000 SOLV, Anton Paar, Graz, Austria).FAMEs were dissolved in heptane and the composition of methyl esters were analyzed on a GC (HP 5890A, Agilent Technologies, Palo Alto, CA, USA) according to the method described by AOCS Ce 1b-89 (1998) with a DB-WAX column (10 m, 0.1 mm, 0.1 µm film thickness, J&W Scientific, Folsom, CA, USA). The initial temperature for the oven was 160ºC and was increased gradually as follows: 160 – 200°C 10.6°C/min (200°C kept for 0.3 min), 200 – 220°C 10.6°C/min (220°C kept for 1 min), 220 -240°C 10.6°C/min (240°C kept for 3.8 min).The determination was made in duplicate on each sample.

2.6. Tocopherol concentration

Oil phase from mayonnaise and lipid extract from milk (after evaporation under nitrogen) were dissolved in heptane and analyzed by HPLC (Agilent 1100 Series, Agilent Technology, Palo Alto, CA, USA) according to the AOCS (1997) method to determine concentrations of tocopherol homologues in the samples. The determination was made in duplicate on each sample.

2.7. Analysis of primary oxidation products, PV

Peroxide value in oil phase obtained from mayonnaise and lipid extracts from milk were assessed according to the method described elsewhere (Shantha & Decker, 1994) based on the formation of an iron-thiocyanate complex. The determination was made in duplicate on each sample.

2.8. Analysis of secondary oxidation products, volatiles

Volatile compounds were collected on Tenax GR packed tubes by dynamic headspace. The extraction of the volatile compounds was done in 4 g of sample, heated at 60 ºC for mayonnaise samples and at 45 ºC for milk samples, during 30 minutes with a nitrogen flow of 150 mL/min. For the mayonnaise sample, volatile acids were removed by KOH during the headspace collection as described by Hartvigsen et al. (2000). The trapped volatiles were desorbed by using an ATD-400 automatic thermal desorber. The transfer line of the ATD was connected to an Agilent 6890 (Palo Alto, CA, USA) gas chromatograph equipped with a HP 5973 mass selective detector. Chromatographic separation of volatile compounds was performed on a DB1701 column (30m x ID 0.25mm x 1µm film thickness, J&W Scientific, Folsom, CA, USA). The oven program was as follows: the initial temperature was 45ºC and was kept for 5 minutes, then the temperature was increased by 1.5ºC/min to 55ºC and then by 2.5ºC/min to 90ºC. Finally, the temperature was increased by 12ºC/min to 220ºC and kept for 4 minutes. Both for mayonnaise and milk the analysis was performed in triplicate and the results are given in ng/g of emulsion.

The quantification of the different volatiles was done by the use of a calibration curve prepared from external standards. Solutions with external standards at different concentrations were prepared and added to a fresh mayonnaise or milk samples prepared with neither fish oil nor antioxidant. Then, the volatiles were collected in the same way as for the samples.

2.9. Statistics

The results obtained was analyzed by two way ANOVA (GraphPad Prism Version 4.01, GraphPad Software,Inc). Bonferroni multiple comparison post-test was used to determine differences between samples or storage times. The significance level used was $p<0.05$. When a significant difference was observed between samples, they are denoted with different superscripts in the text. The lag phase for the treatments in the different oxidation parameters was defined as no significant difference with time 0.

3. Results and discussion

3.1. Fatty acid composition in fish oil enriched mayonnaise and milk

The FAME composition of samples from both experiments was determined at the beginning and at the end of the storage period. At time 0, mayonnaise's EPA and DHA content ranged between 1.77-1.88 and 2.17-

191 2.45%, respectively, and on day 28 between 1.76-1.99 and 1.96-2.22%, respectively. No significant
192 differences were observed between mayonnaises prepared with the different antioxidants. In addition, there
193 were no significant differences between storage times. Thus, the FAME data did not indicate oxidation of
194 EPA and DHA in mayonnaises during storage. However, it is well known that lipids have to be severely
195 oxidized before changes in fatty acid compositions can be observed.

196 As expected, in all treatments the content of EPA (3.12-3.42%) and DHA (3.82-4.19%) in the different milk
197 emulsions were similar at day 0. At day 12, the DHA content remained almost unchanged (3.65-4.16%) in
198 the different emulsions. However, a significant decreased content was found in the control sample (from 3.82
199 to 3.65%). In contrast to the DHA content, the EPA content of milk emulsions at day 12 ranged between 2.98
200 and 3.36% and significantly decreased with the storage time in all the treatments with the exception of the
201 milk with dodecyl caffeate.

202 These different results between the fatty acid compositions in the emulsion systems studied could be related
203 to the difference on the droplets characteristics of both emulsions. Mayonnaise oil droplets are known to be
204 bigger than that of milk (Jacobsen et al., 2000; Sørensen et al., 2012) thus EPA and DHA may be located
205 more easily in the inner core of the oil droplet. Besides, milk droplets are negatively charged, and it has been
206 reported that negatively charged droplets may attract positively charged metal ions that may favor the
207 oxidation development.

208 **3.2. Tocopherol content in the fish oil enriched mayonnaise and milk**

209 Four different tocopherol homologues were detected in mayonnaise samples (α-, β-, γ-, and δ-tocopherol).
210 The results for β- and δ-tocopherol are not presented due to the lack of differences. Neither γ-tocopherol nor
211 α-tocopherol content in mayonnaise emulsions was different between treatments at the beginning or at the
212 end of the storage period. However, the amount of both of these tocopherol homologues decreased during
213 storage leading to a final reduction of 20% (in average) of both α- and γ- tocopherol content at day 28 (Table
214 2). Even the double concentration of octyl caffeate (200μM) in the mayonnaise led to the same consumption
215 of both tocopherol homologues as in the other mayonnaise (antioxidants at 100 μM and control). Finally,
216 although there were no significant differences between treatments at the end of the storage period, the
217 control mayonnaise and the mayonnaise with CA had a slightly smaller decrease in tocopherol content than

the rest of the treatments. This was particularly the case for γ- tocopherol. Mayonnaises had high oil content and therefore a remarkable amount of tocopherol homologues. The reduction in tocopherol content along the storage time was likely due to tocopherol acting as an antioxidant by donating an hydrogen to prevent oxidation of the lipids, although there might had been interactions between tocopherol and caffeates, such as regeneration of tocopherol by caffeic acid.

The reduction potential for tocopheryl radicals (α-TO•) is reported to be slightly lower than that of caffeic acid o-semiquinone (Caf-O•) E_v 0.48 and E_v 0.54, respectively (Laranjinha & Cadenas, 1999). According to the reduction potentials, the regeneration of tocopherol by caffeic acid is thus not thermodynamically feasible. However, it has been observed several times that caffeic acid can regenerate tocoperol (Iglesias, Pazos, Andersen, Skibsted, & Medina, 2009; Laranjinha, Vieira, Madeira, & Almeida, 1995; Medina et al., 2012). Furthermore, a synergistic protection by a combination of tocopherol, caffeic acid and ascorbic acid against free radicals in SDS micelles has been observed from EPR experiments. It was proposed from the results that tocopherol in the hydrophobic phase was regenerated by caffeic acid at the interphase and finally caffeic acid was regenerated by ascorbic acid in the water phase (Laranjinha & Cadenas, 1999). In the present study, mayonnaise was produced with lemon juice; hence, the mayonnaise contained ascorbic acid. Thus, it is suggested that the slightly smaller decrease in tocopherol content was due to regeneration of tocopherol by ascorbic acid in mayonnaise without antioxidant and in the mayonnaise with caffeic acid by regeneration of tocopherol by caffeic acid, which was then regenerated by ascorbic acid. Esterification of caffeic acid increased the lipophilicity of these antioxidants. Thus, the caffeates can be expected to be located closer to the oil phase or maybe interacting with the interface and this may prevent ascorbic acid from efficiently regenerating caffeates, whereby they could not regenerate tocopherol to the same extent. In the absence of caffeic acid or caffeates, ascorbic acid was apparently able to regenerate tocopherol to the same extent as when caffeic acid was present. Moreover, the O-H bond dissociation enthalpy depends on the nature of ring substitutions, which may explain the small differences found between caffeic acid and the esters and between each caffeate assayed.

In milk samples, three tocopherol homologues were found (α-, γ- and δ-tocopherol). However, only α- and γ-tocopherol contents were different between treatments or changed with the storage time (Figure 1). At the beginning of the storage time, milk emulsions with no antioxidant or with added caffeic acid had lower α-

tocopherol content than the rest of the emulsions (Figure 1A). Moreover, the content of α-tocopherol in milk emulsions decreased in all samples during storage. However, a higher reduction was observed in milk emulsions with no antioxidant or with caffeic acid added, whereas no differences between the remaining emulsions were observed with respect to reduction in tocopherol levels. No differences were observed in concentration of γ-tocopherol between milk emulsions neither at the beginning nor at the end of the storage time as observed for α-tocopherol. However, the γ-tocopherol content of the different milk emulsions decreased with the storage time (Figure 1B).

Overall, a lower content of tocopherols was found in the milk emulsion with no antioxidant added. The relative decrease in α- and γ- tocopherol content in milk samples (respectively, on average, 28% and 22%) was greater than in mayonnaise samples despite the shorter storage time and lower storage temperature in the milk samples. Similarly to what happens for EPA and DHA in mayonnaises, the bigger droplets of mayonnaise may allow the dispersion of the tocopherol homologues into the core of the oil droplet, thus avoiding their contact with the water soluble pro-oxidants present in the system. Besides, the negatively charged droplets of milk may attract the positive charged metal ions thus favoring the tocopherol behavior as antioxidant by donating a hydrogen.

3.3. Primary oxidation products – Peroxide Value

PV was measured in mayonnaise (Figure 2A) and milk (Figure 3A) emulsions during storage in order to monitor the hydroperoxides development. For almost all samples (mayonnaise and milk), the PV did not increase from the beginning of the storage period thus presenting a lag phase. However, the length of this lag phase in each experiment was different between treatments.

In mayonnaise samples, butyl caffeate and octadecyl caffeate resulted in longer lag phases (9 days) whereas no antioxidant addition or octyl caffeate at 200 μM resulted in shorter lag phase (3 days). The rest of the mayonnaise samples (Mayo_CA, Mayo_CAC1, Mayo_CAC8 and Mayo_CAC12) had a lag phase of 6 days. Despite these differences in the lag phase, at the end of the storage period (28 days), the PV of the different mayonnaise samples decreased as follows: Mayo_CONTROL^{a} ≥ Mayo_CA^{a} ≥ Mayo_CAC1^{a} ≥ Mayo_CAC18^{a} ≥ Mayo_CAC8^{ab} ≥ Mayo_CAC12^{b} > Mayo_CAC8 200^{c} ≥ Mayo_CAC4^{c}.

Regarding the lag phase in milk emulsions, the only one with no lag phase was the emulsion without antioxidant added. The rest of the milk emulsions presented a lag phase of 3 days for milk emulsion with caffeic acid, 6 days for those with hexadecyl and eicosyl caffeate added and 9 days for the milk emulsion with dodecyl caffeate added. The lag phase could not be defined in milk emulsions with methyl, butyl and octyl caffeates added since there was no significant increment in PV during the storage period.

The rate of lipid hydroperoxides development in milk did not follow the same trend as in the mayonnaise. At the final storage time (12 days), the PV of milk emulsions decreased in the following order: Milk_CONTROL^{a} > Milk_CAC20^{b} ≥ Milk_CA^{b} ≥ Milk_CAC16^{bc} ≥ Milk_CAC12^{cd} ≥ Milk_CAC8^{de} ≥ Milk_CAC4^{e} ≥ Milk_CAC1^{e}.

Overall, these findings supported the idea that the length of the alkyl chain esterified to caffeic acid influenced the development of peroxides in each emulsion system differently. In mayonnaise, the caffeates with medium alkyl chain (octyl, dodecyl and butyl caffeate) delayed the onset of the primary oxidation most efficiently. Conversely, it seems that for milk the PV remained lower with the shorter chain caffeates (i.e. methyl caffeate) when compared to the medium chain caffeates (octyl and butyl caffeate).

3.4. Secondary oxidation products – Content of volatile Compounds

The formation of secondary volatile oxidation products in mayonnaise and milk emulsions were evaluated by dynamic headspace. Although five different volatiles were measured in both of the stored emulsions, only three of them (1-penten-3-one, 1-penten-3-ol and 2,4-heptadienal) are shown for mayonnaise (Figure 2) and milk (Figure 3) emulsions. These volatiles were selected because they illustrate the general trend in the development of volatile compounds in these two emulsion systems during storage. In addition, these compounds are known to represent the decomposition of n-3 PUFA and to have impact on the development of fishy off-flavour (Venkateshwarlu, Let, Meyer, & Jacobsen, 2004).

Mayonnaise emulsions with no antioxidant and those with caffeic acid added had a lag phase of 6-9 days for the development of 1-penten-3-one, whereas the rest of mayonnaise emulsions showed a lag phase of 9-12 days (figure 2B). After this period the concentration of 1-penten-3-one increased in all emulsions up to 28 days of storage. At day 28 the concentration of 1-penten-3-one in the mayonnaise decreased in the following order: Mayo_CA^{a} ≥Mayo_CAC1^{a} ≥ Mayo_CONTROL^{a} ≥ Mayo_CAC8^{a} > Mayo_CAC18 ^{b} ≥ Mayo_CAC4^{b} > Mayo_CAC8 200^{c} ≥ Mayo_CAC12^{c}.

In comparison to 1-penten-3-one, the 1-penten-3-ol lag phase was longer even when the mayonnaise did not contain antioxidant (Figure 2C). Whereas almost all mayonnaises had 12 days of lag phase, those with caffeic acid, methyl caffeate and octyl caffeate (at 200μM) added had a lag phase of 9 days. However, the rate for the development of 1-penten-3-ol was faster than that found for the 1-penten-3-one. Although the ranking of 1-penten-3-one is a bit different than that found for 1-penten-3-ol, again the lowest concentration of this volatile was found in Mayo_CAC12 and MAYO_CAC8 200 followed by Mayo_CAC4.

Opposite to what was found for the previous described volatiles, 2,4-heptadienal increased with storage time from the beginning of the storage period meaning that a lag phase did not exist (Figure 2D). As for the other two volatiles, the lowest concentration of 2,4-heptadienal was found for Mayo_CAC12, Mayo_CAC4 and MAYO_CAC8 200. Similar to the observation of 1-penten-3-ol concentration in the mayonnaise, the highest concentration of 2,4-heptadienal was found in Mayo_CONTROL followed by Mayo_CA.

Overall, those emulsions without antioxidant or containing the native phenolic had higher concentrations of volatile compounds than mayonnaises containing caffeates. The most effective caffeates added to fish oil enriched mayonnaise were those with short to medium chain (butyl, octyl and dodecyl). Further increase of the alkyl chain length followed a collapse in the antioxidant capacity of the esterified phenolic compound, thus supporting the cut-off effect theory. Therefore, it can be affirmed that the polar paradox does not fully explain the behavior of the antioxidants in these mayonnaises. Moreover, the cut-off theory seems a better approach to explain the oxidation in this complex matrix. Besides, the trend observed for PV and volatiles development in the different mayonnaises was similar, meaning that the caffeates were able to delay formation of both primary and secondary oxidation products in mayonnaise.

The volatiles found in the milk storage experiment are shown in Figure 3B-D. In general, the concentration of 1-penten-3-one increased until day 9 and thereafter the concentration decreased at different rates between different treatments. Milk emulsions containing caffeic acid esterified with longer alkyl chains (dodecyl, hexadecyl and eicosyl caffeate) presented a lag phase of 3 days before 1-penten-3-one started developing in these emulsions. The concentration of 1-penten-3-one in milk emulsions with methyl and butyl caffeates added did not significantly increase during storage time. For the remaining milk emulsions there was no lag phase as 1-penten-3-one developed from the beginning of the storage period. The ranking of the emulsions according to the concentration of 1-penten-3-one before the decrease in concentration (Day 9) was as

follows: Milk_CONTROL[a] > Milk_CA[b] ≥ Milk_CAC16[b] > Milk_CAC12[c] > Milk_CAC20[d] ≥ Milk_CAC8[d] > Milk_CAC4[e] ≥ Milk_CAC1[e]. The decrease in concentration of 1-penten-3-one observed at the end of the storage period might be due to a reduction of this volatile to 1-penten-3-ol either by the antioxidant or by other components in the milk emulsion. Moreover, the reduction of 1-penten-3-one after development has been observed in another study (Sørensen et al., 2012).

1-penten-3-ol and 2,4-heptadienal measured in all milk emulsions had a lag phase period, which is illustrated in Fig. 3C and D. The pattern of these volatiles was similar to that found for 1-penten-3 one. Generally, the highest concentration of volatiles measured over the storage period was in the control milk emulsion. The lag phase for the development of 1-penten-3-ol was 3 days in milk with no antioxidant and with caffeic acid added. In milk emulsions with dodecyl, hexadecyl and eicosyl caffeate added, the lag phase was 6 days whereas in milk with octyl caffeate added the lag phase was 9 days. Similar to what was found for the concentration of 1-penten-3-one, milk emulsions with methyl and butyl caffeates added did not increase significant in their 1-penten-3-ol content during the storage period.

The duration of the lag phase for the development of 2,4-heptadienal was different from that of the previous described volatiles. Here, the milk emulsions with octyl and hexadecyl caffeate added did not show a lag phase whereas the samples with no antioxidant and those with dodecyl and eicosyl caffeates added had a lag phase of 3 days. Finally, milk emulsions with methyl and butyl caffeate added had a lag phase of 9 days. After the induction period, the concentration of 1-penten-3-ol and 2,4-heptadienal increased during storage. At day 12, the ranking pattern of 1-penten-3-ol and 2,4-heptadienal in the different milk emulsions were similar to that observed for 1-penten-3-one. Thus, the milk emulsions with less volatile content were those with methyl and butyl caffeates added and the milk with higher volatile content was the milk emulsion without antioxidant added.

Similar results were achieved when assessing the effectiveness of the caffeates in o/w emulsions with citrem (Sørensen et al., 2013) where both caffeic acid and methyl caffeate protected samples from development of both primary and secondary oxidation.

In general, the pattern of development of volatile compounds was similar to that found for the PV development in both emulsion systems.

354 The effectiveness of caffeates added to fish oil enriched milk was highest when the methyl and butyl alkyl
355 chain were esterified to the caffeic acid moiety. However, the antioxidant capacity decreased with further
356 increase of the alkyl chain length esterified to the phenolic compound, thus supporting the cut-off effect
357 theory. From these results, it is reasonable to assume that the non-linear theory explains more precisely the
358 behavior of the antioxidants in food emulsions than the polar paradox hypothesis, which seems to be too
359 simple to fully explain the oxidation in these complex matrixes.

360 **3.5. Difference between efficacy of caffeates in mayonnaise and milk emulsions**

361 The oxidation results of both storage experiments indicated that mayonnaise and milk emulsions without
362 antioxidants added generally oxidized faster than emulsions with caffeic acid or caffeates. Interestingly, the
363 antioxidant capacity of caffeates was different in each emulsion system. Medium chain (butyl, octyl and
364 dodecyl) caffeates were more effective in delaying the onset of oxidation in mayonnaise emulsions enriched
365 with fish oil whereas in fish oil enriched milk emulsions caffeates with shorter alkyl chain (methyl and butyl)
366 were more efficient.

367 Laguerre et al. (2008) developed an assay to assess antioxidant effectiveness in an emulsion system
368 denominated conjugated autoxidizable triene (CAT) assay. The CAT values for the different caffeates had
369 been assessed by Sørensen et al. (2013) and the most effective ones were octyl caffeate and dodecyl
370 caffeate , whereas the antioxidant properties of the caffeates collapsed beyond twelve carbon chain length
371 (C12). This behavior is similar to that found for the mayonnaise emulsions; however, in mayonnaises there
372 were almost no differences between C4, C8 and C12. Nevertheless, these findings do not explain the
373 antioxidant efficiency of these phenolipids in milk or simple o/w emulsions (using citrem as emulsifier)
374 (Sørensen et al., 2013) .

375 Laguerre et al. (2009) was the first to introduce the cut-off effect theory. Recently a putative mechanism of
376 action for the cut-off effect in o/w emulsions has been pointed out, namely: “reduced mobility”,
377 “internalization” and “self-aggregation” hypotheses (Laguerre et al., 2013). These three hypotheses focus on
378 what happens beyond the cut-off, when the antioxidant capacity suddenly collapses. Below the critical chain
379 length, it is assumed that the antioxidants are not close enough to the interface where oxidation is occurring.

These three hypotheses of mechanism of action of the cut-off effect theory will be driven by the characteristic of the emulsion which in our case are complex matrices (mayonnaise and milk). Fish oil enriched mayonnaise and milk emulsions contain proteins and other minor components (minerals, sugars, natural antioxidants and chelators, etc.) that are water soluble and are not present in simple o/w emulsions. All these components can interact with the antioxidants in the emulsion system and affect their location and thus their effectiveness (McClements & Decker, 2000). Furthermore, several authors (McClements & Decker, 2000; Shahidi & Zhong, 2011; Sørensen et al., 2008) suggested that, besides these above mentioned components, the emulsifier used in the emulsified medium can interact with the antioxidants added. The antioxidant capability will be reduced by the competition with emulsifiers for their localization at the interface.

The reduced mobility theory points out that the mobility of the lipophilic antioxidant decreases as its alkyl chain length increases, thus decreasing its ability to move toward the numerous oxidation sites. The higher oil content of mayonnaise (>80%) vs. milk (1.5%) makes the mayonnaise a more viscose and non-polar medium than milk emulsion. The more viscose mayonnaise will make the diffusion of the antioxidants in the medium more difficult, decreasing its ability to move toward the numerous oxidation sites. Thus, those medium chain caffeates, which may be located near the water-oil interface would be much more efficient than any other caffeates that would need to move to the site of oxidation. Conversely, as the viscosity of the milk compared with the mayonnaise is much lower, the mobility of the different caffeates in milk emulsion would not have been so affected. Besides, the more polar milk emulsion system may allow the more polar caffeates to move easier in the milk than in mayonnaise emulsions, which supports the fact that the short chain caffetaes were effective antioxidants in milk emulsions, whereas these antioxidants showed no antioxidant effect in mayonnaise.

The second hypothesis, the internalization, states that increasing the hydrocarbon chain from a medium to long chains could drive the antioxidant away from the interface into the emulsion droplet. Again, the higher oil content of mayonnaise will be able to “host” more long chain caffeates than the droplets of milk, which probably will led them to self-aggregate (third hypothesis) in the water phase of the milk emulsion. A method for antioxidant location in such complex matrixes would be really valuable for understanding how the chain length affects the location of the antioxidant in the different food systems.

Many other differences between the two systems can have affected the antioxidant properties of the caffeates such as the tocopherol content, the protein emulsifiers, pH and oil droplet characteristics (charge, size and composition) of milk and mayonnaise. Regarding the actual difference in composition of both systems, the tocopherol content of mayonnaise was higher than that of milk due to its 64% content of rapeseed oil. It has been reported that the addition of rapeseed oil to emulsions enriched with fish oil protected them from oxidation due to its high tocopherol content (Let, Jacobsen, Pham, & Meyer, 2005). In the same study, it was proven that it is not only the tocopherol content, but also the matrix in which the tocopherol was present that protected emulsions from oxidation.

Emulsion systems had several differences regarding their droplet size, charges and surface composition. Mayonnaise emulsions are known to have bigger oil droplets than milk emulsions (Jacobsen et al., 2000; Sørensen et al., 2012). Both bigger oil droplets and higher oil content in mayonnaise comparing with milk emulsions may affect the location of the caffeates in the different systems. However, this should be further studied before any conclusion can be drawn.

Besides that, mayonnaise oil droplets are positively charged, whereas milk oil droplets are negatively charged. Differences in oil droplets charge are due to the protein composition of the interface and the pH found in the medium (<4.2 for mayonnaise and 6.7 for milk). It has been reported that negatively charged droplets may attract positively charged metal ions which may favour lipid oxidation development as has been stated before. Both PV and volatile concentration on mayonnaise emulsions are higher than that observed for milk emulsions. However, this fact is related to the higher amount of oil in the mayonnaise emulsions. Mayonnaises contain more than 40 times more oil than milk emulsions. Thus, based on oil content, the highest oxidation was observed for the milk with negative charged droplet.

Mayonnaise droplet interface would be composed of a lecithin-protein complex (phosvitin, lipovitellin, livetin) and LDL (egg yolk plasma and granules), whereas milk droplets interface would be composed of caseins, whey proteins and milk phospholipids. Panya and coworkers (Panya et al., 2012) recently pointed out that the effectiveness of the rosmarinates can be influenced by an excess of emulsifier in o/w emulsions. It was observed that the non-polar eicosyl rosmarinate was less effective at inhibiting lipid oxidation in o/w emulsions than rosmarinate esters with shorter fatty alkyl chains. However, in the presence of surfactant micelles, the antioxidant activity of the eicosyl rosmarinate was significantly increased while the antioxidant

435 effectiveness of butyl and dodecyl rosmarinates slightly decreased. The explanation for the observation was
436 that the eicosyl rosmarinate was located in the inner core of the oil droplet, and that the excess of emulsifier
437 formed micelles that were able to modify the location to the interface where the oxidation is initiated. In our
438 mayonnaise and milk emulsions there was an excess of emulsifier (either egg yolk in mayonnaise or milk
439 proteins in milk) that may have influenced the antioxidants differently in the two food systems. This may
440 explain why a broader range of critical chain length was observed compared to one critical chain length with
441 the more simple emulsion system (measured through CAT assay). Besides, the complexity of real food
442 samples may contribute to the more unclear effect in comparison with simpler o/w emulsions found in the
443 literature (Laguerre et al., 2009).

444 Finally, the interaction between tocopherol and caffeates cannot be avoided, in both systems the reduction of
445 the α-tocopherol with the storage time was different depending on the caffeate added. This may indicate that
446 some tocopherol regeneration may have taken place in the systems. However, this regeneration may
447 depend on the matrix. Thus, α-tocopherol reduction in mayonnaise was smaller when no antioxidant or
448 caffeic acid was added alone, whereas in milk the opposite behavior occurred.

449 In the review of Laguerre et al., 2013, they raise the question as to whether the critical chain length is
450 constant or variable, questioning if it would depend on the system studied or not. In our case, it is clear that
451 the critical chain length depends on the system, as the cut-off effect was different in mayonnaise and milk
452 emulsions. Therefore, the use of different phenolipids to minimize oxidation is a complex issue that requires
453 the study of the effectiveness of phenolipids in each particular food emulsion system.

454 **4. Conclusion**

455 In conclusion, caffeic acid and its esters acted as antioxidants in both mayonnaise and milk emulsions
456 enriched with fish oil. In both emulsion systems, the derivatized caffeates showed a higher antioxidant
457 capacity than the native phenolic compound, caffeic acid. Interestingly, the effectiveness of the caffeates
458 was different in each matrix. Caffeates with short to medium chain (C4, C8 and C12) were effective in
459 mayonnaise enriched with fish oil, whereas those with shorter chain (C1 and C4) were more effective as
460 antioxidants in milk enriched with fish oil. Thus, optimal alkyl chain length for phenolipids depends on the
461 matrix studied. Moreover, this suggests that it is not possible to extrapolate the optimal chain length from one

system to another one. Results obtained from milk or from CAT assay do not allow the prediction of results obtained in mayonnaise, and vice versa. Each system should be tested.

The effectiveness of caffeates in real food systems is really promising, thus future work should be performed in this area to fully understand the underlying mechanism. Furthermore, partitioning of the different phenolipids in complex emulsion systems will help to explain and predict the behavior of those antioxidants in real food products.

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566

567 **Figure 1:** α-tocopherol (A) and γ-tocopherol (B) content of milk emulsions along the storage period. For
568 interpretation of code names please refer to Table 1. Error bars indicate SD of the measurements (n=2).

569

570 **Figure 2:** Mayonnaise emulsions concentration of peroxides measured as PV [meq. peroxides/kg oil] (A),
571 concentration of 1-penten-3-one (B), 1-penten-3-ol (C) and 2,4-heptadienal (D) [ng/g mayonnaise] in the
572 different fish oil enriched mayonnaises during storage time. Error bars indicate SD of the measurements (n
573 =2 for PV and n=3 for volatiles compounds).

574

575 **Figure 3:** Milk emulsions concentration of peroxides measured as PV [meq. peroxides/kg oil] (A),
576 concentration of 1-penten-3-one (B), 1-penten-3-ol (C) and 2,4-heptadienal (D) [ng/g mayonnaise] in the
577 different fish oil enriched mayonnaises during storage time. Error bars indicate SD of the measurements (n
578 =2 for PV and n=3 for volatiles)

579

580

581

582 **Table 1:** Experimental design

Antioxidant applied	Sample code	Concentration [µM]
Control	Mayo_CONTROL	-
Caffeic acid	Mayo _CA	100
Methyl caffeate	Mayo _CAC1	100
Butyl caffeate	Mayo _CAC4	100
Octyl caffeate	Mayo _CAC8	100
Octyl caffeate	Mayo _CAC8 200	200
Dodecyl caffeate	Mayo _CAC12	100
Octadecyl caffeate	Mayo _CAC18	100
Control	Milk_CONTROL	-
Caffeic acid	Milk _CA	100
Methyl caffeate	Milk _CAC1	100
Butyl caffeate	Milk _CAC4	100
Octyl caffeate	Milk _CAC8	100
Dodecyl caffeate	Milk _CAC12	100
Hexadecyl caffeate	Milk _CAC16	100
Eicosyl caffeate	Milk _CAC20	100

583 **Table 2:** α-tocopherol and γ-tocopherol content in mayonnaise at day 0 (mean value ± SD, n=2), and the
584 final percentage reduction at day 28.

	Day 0		*Reduction*	
Code	**α-tocopherol [µg/g oil]**	**γ-tocopherol [µg/g oil]**	**α-tocopherol %**	**γ-tocopherol %**
Mayo_CONTROL	218.1 ± 0.2	269.1 ± 1.5	17.4	14.9
Mayo_CA	219.8 ± <0.01	267.1 ± 4.3	17.1	14.6
Mayo_CAC1	222.7 ± 0.3	269.8 ± 1.8	22.0	20.6
Mayo_CAC4	221.4 ± 3.4	269.1 ± 1.1	20.3	21.7
Mayo_CAC8	217.3 ± 1.0	266.0 ± 1.0	19.1	19.5
Mayo_CAC8 200	222.4 ± 0.7	274.7 ± 1.3	21.2	23.5
Mayo_CAC12	217.9 ± 2.5	273.6 ± 4.7	19.4	22.1
Mayo_CAC18	223.5 ± 2.9	276.2 ± 1.6	22.3	21.9

585 For interpretation of code names please refer to Table 1.

586

587

Figure 1

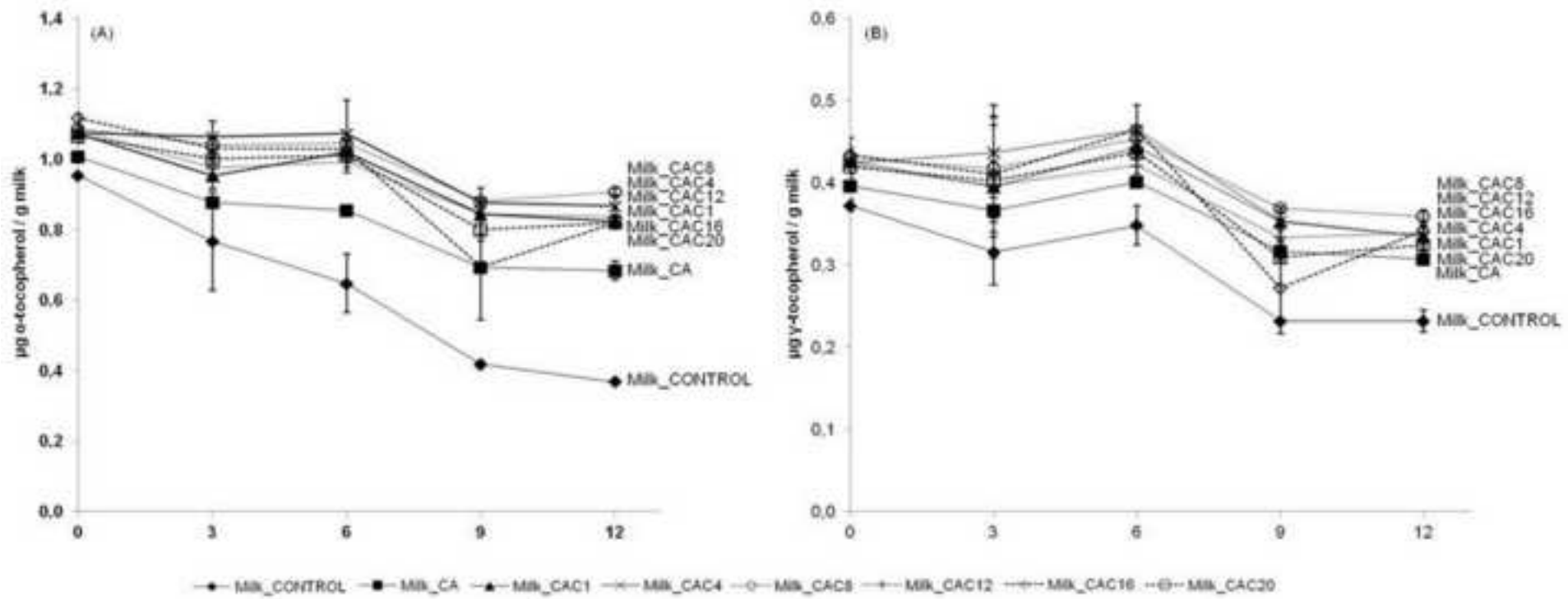

Figure 2

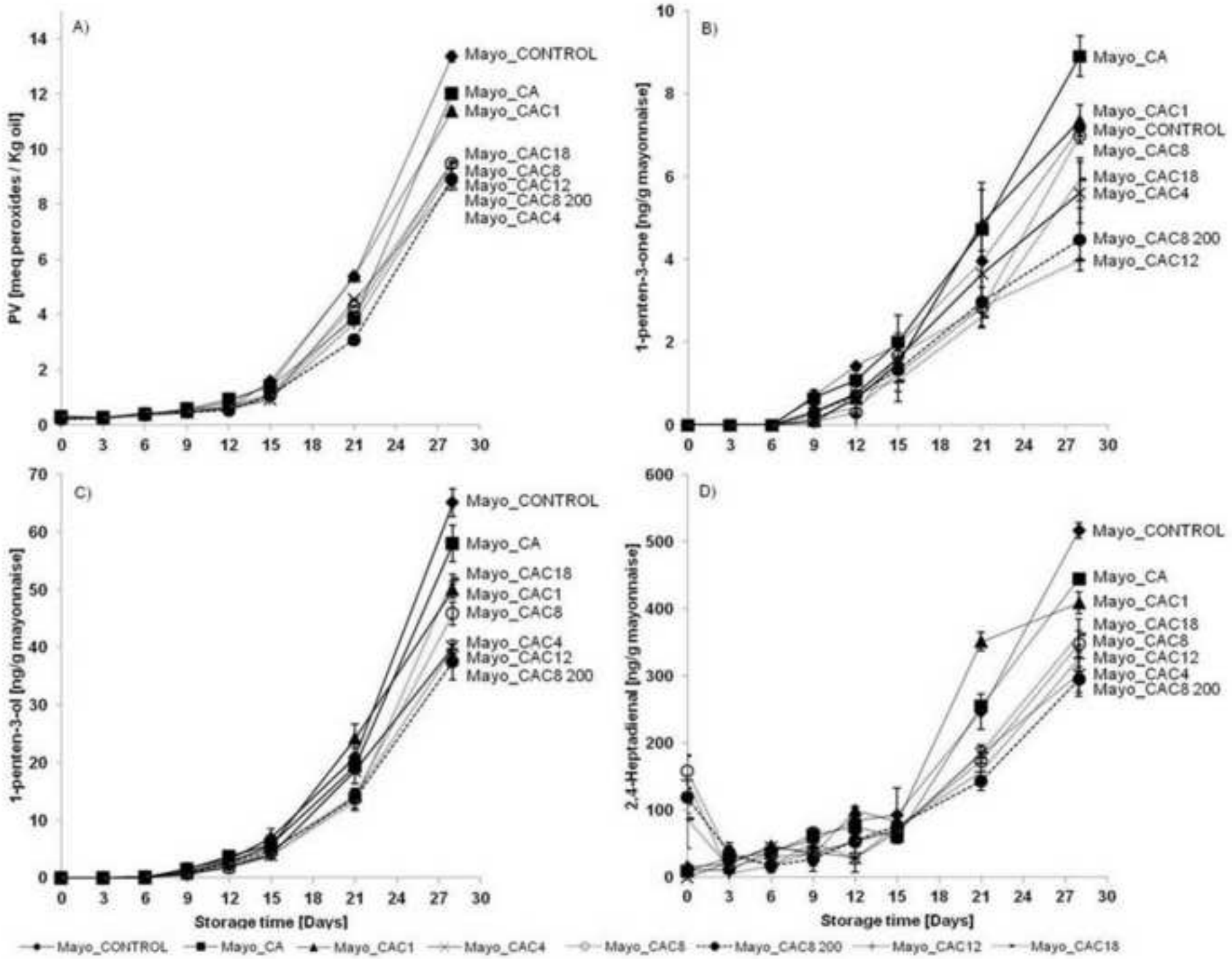

Figure 3