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ORIGINAL PAPER

# The Efficacy of Compounds with Different Polarities as Antioxidants in Emulsions with Omega-3 Lipids

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**Abstract** According to the so-called polar paradox hypothesis, the efficacy of an antioxidant in emulsions is highly affected by its polarity and thereby location in the different phases. However, other factors also affect the efficacy of antioxidants in multiphase systems. The aim of this study was to evaluate the efficacy of antioxidants [ascorbic acid, ascorbyl palmitate, ascorbyl CLA and CLA (conjugated linoleic acid)] with different polarities in two different emulsion systems: o/w emulsion (5% oil) and w/o emulsion (98% oil) stabilized with citrem and PGPR, respectively. The efficacy of the antioxidants was compared to their partitioning in an o/w emulsion system and to results obtained from different antioxidant assays: iron reducing power, chelating activity and radical scavenging activity. For the w/o emulsions the efficacy of the antioxidants followed the polar paradox hypothesis: ascorbyl palmitate = ascorbyl CLA > ascorbic acid ≥ CLA > reference. For the o/w emulsion the antioxidative effects were not in accordance with the polar paradox. In the beginning of the storage, ascorbyl palmitate and ascorbic acid were most efficient, however in the end they acted as prooxidants. Ascorbyl CLA was located at the interface but was inactive as an antioxidant. This may be due to impurities or interaction with citrem.

**Keywords** O/W emulsion · W/O emulsion · Lipid oxidation · Antioxidant activity and partitioning

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## Introduction

Healthy n-3 long chain polyunsaturated fatty acids (PUFA) in fish oil, EPA and DHA, are currently added to a number of different food systems as functional ingredients. These lipids are highly susceptible to lipid oxidation and therefore protection against oxidation, e.g. by antioxidant addition is necessary.

According to the so-called polar paradox hypothesis, the efficacy of antioxidants in multiphase systems are highly affected by the polarity of the antioxidants and thereby the distribution in the different phases of multiphase systems [1, 2]. Therefore, amphiphilic antioxidants have previously been suggested to have better efficacy than lipophilic or hydrophilic antioxidants in multiphase systems [2]. In addition, earlier work of Frankel and co-workers [1] on the antioxidant effect in bulk oil versus emulsion showed that ascorbyl palmitate was more efficient in 10% o/w emulsions than ascorbic acid. The reverse trend was evident in bulk oil [1]. Thus, we hypothesize that the antioxidative properties of hydrophilic compounds such as ascorbic acid in emulsions may also be improved by esterifying hydrophilic antioxidants with other fatty acids such as CLA (conjugated linoleic acid).

Ascorbyl palmitate is an antioxidant, which has been tested and used in several food systems [3] whereas the

antioxidative effect of ascorbyl CLA has not previously been investigated. Earlier studies have shown a weight reducing effect of CLA as well as antioxidative [4, 5] and anticarcinogenic effects in vitro and in animal studies [6]. However, the antioxidative properties reported for CLA conflicted with other reported results, which showed prooxidative effects of CLA [7].

Several reports on lipophilized ascorbic acid have indicated antioxidative effects of the esters in different systems [8]. Thus, a combination of ascorbic acid and CLA might have a similar antioxidative activity compared with ascorbyl palmitate.

Emulsions can either be o/w or w/o emulsions. The initial step in lipid oxidation in emulsions has been suggested to take place at the interface between the oil and water phases [9]. Differences in the efficacy of amphiphilic antioxidants in the two systems are hard to anticipate. A few reports are available on the effects of antioxidants in w/o emulsions and to our knowledge no direct comparison of the efficacy of the same antioxidant in o/w and w/o emulsions has been reported.

Lipid hydroperoxides are more polar than lipids and therefore more water soluble than lipids. Especially lipid hydroperoxides from EPA and DHA are more polar [10] since more than one peroxyl group can be attached to the same fatty acids. Hence, it is expected that peroxyl radicals to some degree will be present at the water–lipid interface. Moreover, hydroxyl radicals might be present in the water phase. Therefore, it may be anticipated that hydrophilic antioxidants will offer better protection in the w/o system than in the o/w system. Moreover, in the w/o emulsion hydrophilic antioxidants will be present in a relatively higher concentration in the disperse water phase than in the continuous water phase in the o/w emulsion provided that the disperse phase constitutes approximately the same volume in the two systems. The higher concentration of hydrophilic antioxidants in the water phase in the w/o emulsion may imply that antioxidant will be located closer to the interface in the w/o emulsion than in the o/w emulsion and this could in turn lead to a better effect.

To investigate our hypothesis and to improve our understanding of the antioxidant effects in w/o versus o/w emulsions, the effect of four different antioxidants with different polarities was evaluated in two model systems: o/w emulsion (5% oil) with citrem as an emulsifier and w/o emulsion (98% oil) with PGPR (polyglycerol polyricinoleat) as emulsifier, both at pH 7. The development of hydroperoxides and secondary volatile oxidation products were measured. In addition the antioxidative effects of the antioxidants (ascorbyl palmitate, ascorbyl CLA and ascorbic acid) were compared with their partitioning in a system resembling the o/w emulsions. Furthermore, the antioxidative mechanism was characterized by different antioxidant assays: iron reducing power, chelating activity and radical scavenging activity (DPPH).

## Materials and Methods

### Materials

The mixture of fish oil and rapeseed oil (1:1) without added antioxidant was supplied by Maritex Norway (subsidiary of TINE BA, Norway). This oil had an initial PV <0.1 mequiv peroxides $kg^{-1}$ oil, tocopherol content of 274 mg $\alpha$-tocopherol, 30 mg $\beta$-tocopherol, 172 mg $\gamma$-tocopherol and 9 mg $\delta$-tocopherol $kg^{-1}$ oil, and the fatty acid composition was as follows: 14:0, 1.5%; 16:0, 7.0%; 16:1, 4.2%; 18:0, 1.9%; 18:1, 42.0%; 18:2, 10.2%; 18:3, 4.6%; 18:4, 1.1%; 20:1, 6.6%; 20:5 (EPA), 4.5%; 22:1, 3.2% and 22:6 (DHA), 5.9%. The total percentages of n-3 and n-6 in the oil were 11.5 and 14.6%, respectively.

The emulsifiers polyglycerol polyricinoleate (PGPR) and citrem LR 10 Extra (citric acid ester of mono- and diglyceride) without antioxidants were supplied by Palsgaard A/S (Juelsminde, Denmark) and Danisco A/S (Grindsted, Denmark), respectively. Ascorbic acid was obtained from Merck (Darmstadt, Germany), ascorbyl palmitate was obtained from Sigma-Aldrich (Steinheim, Germany), CLA (Tonalin® FF80) was obtained from Cognis (Monheim, Germany). Ascorbyl CLA was synthesized at the Department of Molecular Biology, Faculty of Science, Aarhus University. Chemicals were purchased from Merck (Darmstadt, Germany) and external standards for identification and quantification of secondary volatile oxidation products were all from Sigma-Aldrich (Steinheim, Germany). All solvents were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland) or Fisher Scientific (Fair Lawn, USA).

### Antioxidant Activity Assays

#### *Iron Chelating Activity*

Ferrozine is an iron chelating compound, which develops an intensively colored complex with an absorption maximum at 562 nm when free ferrous iron is chelated by the ferrozine molecule. Briefly, antioxidant solutions (0–200 μmol $L^{-1}$) were made up to a volume of 3.7 mL with distilled water. Due to solubilization problems with CLA in water, CLA was dissolved in small amounts of methanol and then diluted with water. Ferrous chloride (2 mmol $L^{-1}$, 0.1 mL) was added and after 3 min the reaction was inhibited by the addition of a ferrozine solution (5 mmol $L^{-1}$, 0.2 mL). The mixture was shaken vigorously and left at room temperature for 10 min, whereafter the absorbance was measured at

562 nm (UV mini 1240 Shimadzu, Kyoto, Japan). The chelating activities (%) of the antioxidants were calculated as follows: $Fe^{2+}$chelating activity $= (\frac{A_0 - A_s}{A_0}) \times 100$, where $A_0$ is absorbance of control (only iron and ferrozine) and $A_s$ is absorbance of sample minus absorbance of sample blank. Triplicate measurements were performed and EDTA was included as a positive control.

*Radical Scavenging Effect*

DPPH is a free radical with a purple color with an absorption maximum at 517 nm. When the odd electron of DPPH becomes paired the color changes to yellow. The antioxidant (0–200 μmol $L^{-1}$ in methanol) was mixed with a 0.1 mmol $L^{-1}$ methanolic solution of DPPH (1:4, v/v). Absorbance was measured after 30 min (ambient temperature, darkness) at 517 nm (UV mini 1240 Shimadzu, Kyoto, Japan). The results were reported as percent inhibition: inhibition $[\%] = (1 - \frac{A_s}{A_0}) \times 100$, where $A_s$ is absorbance of DPPH after reaction with antioxidant minus absorbance of antioxidant in methanol and $A_0$ is absorbance of DPPH in methanolic solution. Triplicate measurements were performed and BHT was included as a positive control.

*Reducing Power*

Reduction of $Fe^{3+}$ to $Fe^{2+}$ can be measured spectrophotometrically at 700 nm, since $Fe^{2+}$ forms a colored complex with cyanide. An aliquot of sample (1 mL, 0–200 μmol $L^{-1}$ antioxidant) was mixed with 0.2 mol $L^{-1}$ phosphate buffer (pH 6.6, 2.5 mL) and 30 mmol $L^{-1}$ potassium ferricyanide (2.5 mL). The mixture was incubated for 20 min at 50 °C. A 0.6 mol $L^{-1}$ trichloroacetic acid solution (2.5 mL) was added and thereafter the mixture was centrifuged (10 min, 2,000 rpm). The upper layer of the solution (2.0 mL) was mixed with an equal amount of distilled water and 0.4 mL 6 mmol $L^{-1}$ $FeCl_3$, and the absorbance (700 nm) was measured after 10 min. High absorbance equals high reducing power. Triplicate measurements were performed.

Partitioning of Antioxidants

Partitioning of the three different antioxidants (ascorbic acid, ascorbyl palmitate and ascorbyl CLA) in buffer/oil, emulsifier/buffer and in o/w emulsion was measured according to the method described below [11]. Antioxidants were dissolved in methanol and the methanol was evaporated by nitrogen before the different phases were added into the centrifuge tube in the buffer/oil and emulsifier/buffer system.

*Water (buffer)/Oil*

Antioxidant (100 μmol $L^{-1}$), 10 mmol $L^{-1}$ acetate-Imidazole buffer (95%) and oil (5%) to a total amount of 40 g were vigorously mixed with a vortex mixer and thereafter placed overnight at 5 °C in sealed centrifuge tubes in the dark for equilibration of antioxidants between the phases. The next day the oil and water phases were separated by centrifugation (10 min, 210*g*). The oil phases were discarded and the water phase centrifuged once more before analysis of antioxidant concentration. This experiment was performed in triplicate for each antioxidant.

*Emulsifier/Water (Buffer)*

Antioxidant (100 μmol $L^{-1}$), 10 mmol $L^{-1}$ acetate-imidazole buffer (99%) and citrem (1%) to a total amount of 40 g were vigorously mixed with a vortex mixer and thereafter placed overnight at 5 °C in sealed centrifuge tubes in the dark for equilibration of antioxidants between the phases. Thereafter, the buffer was separated from citrem by centrifugation (3 mL, 10 min, 210*g*) in Amicon® Ultra centrifugal filter devices (regenerated cellulose 3,000 MW cut off, Millipore, Carrigtwohill, Ireland). The buffer in the bottom of the tube was collected and the rest of the buffer-citrem solution at the top was discarded every time and the aqueous phase in the bottom was discarded the first three times to saturate the filter. This procedure was repeated six times and the last three buffer fractions were collected for measuring the concentration of antioxidant in the buffer phase. This experiment was performed in triplicate for each antioxidant.

*Emulsions (O/W)*

Antioxidant (100 μmol $L^{-1}$), 10 mM acetate-imidazole buffer (94%), oil (5%) and citrem (1%) to a total amount of 200 g were mixed for 3 min with an Ultra-Turrax (Janke & Kunkel IKA-Labortechnik, Staufen, Germany) and thereafter placed overnight at 5 °C in sealed centrifuge tubes in the dark for equilibration of antioxidants between the phases. The procedure described for the emulsifier/buffer system was also used here to separate the buffer from the emulsion. Two emulsions were performed for each antioxidant with duplicate separation of the aqueous phase from each emulsion.

Concentration of Antioxidants

*Ascorbic Acid*

Ascorbic acid in the buffer was quantified using an enzymatic kit (R-BIOPHARM AG, Darmstadt, Germany).

In two of the partitioning studies: emulsifier/water and the whole emulsion, the buffer fractions obtained by repeated centrifugations after saturation of the filter, were pooled prior to analysis.

*Ascorbyl Palmitate and Ascorbyl CLA*

These compounds were analyzed by HPLC (Agilent 1100 Series, Agilent Technology, CA, USA) with a C18 Thermo Hypersil ODS (250, 4.6 mm) column and using a gradient elution at a flow rate of 1 mL $min^{-1}$. Solvent A was 1% phosphoric acid (v/v) and solvent B was acetonitrile:methanol, 1:1 (v/v). Gradient condition: 0–20 min 80–100% B and 20–25 min 100–80% B. The injection volume was 20 μL. Ascorbyl palmitate and Ascorbyl CLA were measured spectrophotometrically at 250 nm. Ascorbyl palmitate and ascorbyl CLA were quantified by calibration curves of these compounds dissolved in methanol and acetonitrile, respectively.

Emulsion Production for Storage Experiments

It is not possible to use the same emulsifier for production of o/w and w/o emulsions, but the emulsifiers used in the present study were selected to have similarities in their structure (Fig. 1). Since both have a glycerol structure with either citric acid (citrem) and 1 or 2 fatty alkyl group substituted or different degrees of fatty alkyl groups substituted to an *n*th degree polymerized glycerol (PGPR, *n* average >1). The antioxidants ascorbic acid, ascorbic palmitate, ascorbic CLA and CLA (Fig. 2), were added separately in a concentration of 100 μM in the emulsion. The antioxidative effects were evaluated during storage of both o/w and w/o emulsions (37 °C).

*W/O Emulsions*

The water-in-oil emulsion systems consisted of 98% oil (fish oil and rapeseed oil, 1:1), 1% 10 mmol $L^{-1}$ acetate-imidazole buffer (pH 7), 1% PGPR as emulsifier and 100 μmol $L^{-1}$ antioxidant (CLA only 68 μmol $L^{-1}$ due to deviation in purity). Oil with PGPR was heated in a water bath (50 °C, 15 min). Buffer was added within the first min of 2 min prehomogenization with a hand-held homogenizer (M133/1281-0, Biospec Products, Inc., Bartlesville, OK, USA). For emulsification a microfluidizer (Microfluidics, Newton, MA, USA) was used with the settings as follows, a 75-μm chamber, pressure at 9,000 psi and three circulations of the emulsions. During homogenization, ice was used to cover the homogenizer chamber and coil in order to maintain the emulsion temperature around 25 °C. Ascorbic acid, ascorbyl palmitate or ascorbyl CLA was added directly to the continuous phase (oil), whereas CLA was dissolved in methanol and then added to the oil (0.5 mL). The same amount of methanol was added to all the other emulsions. The final emulsion was pipetted into 10-mL GC vials (volume: 1.1 mL equals 1 g of emulsion) capped with aluminium caps with PTFE/silicone septa and stored in the dark at 37 °C. For each sampling day three vials were analyzed once (triplicates) for each type of analysis. For the w/o emulsions, one reference without antioxidant was included.

*O/W Emulsions*

The oil-in-water emulsions consisted of 94% 10 mmol $L^{-1}$ acetate-imidazole buffer (pH 7), 5% oil (fish oil and rapeseed oil, 1:1), 1% citrem as emulsifier and 100 μmol $L^{-1}$ antioxidant (CLA only 68 μmol $L^{-1}$ due to deviation in purity). Similar to the w/o emulsions, the o/w emulsions were pre-emulsified prior to homogenization. For pre-emulsification, the buffer was stirred with an Ultra-Turrax (Janke & Kunkel IKA-Labortechnik, Staufen, Germany) and the oil-citrem solution was added during the first min of the 2 min total mixing. The pre-emulsion was then homogenized at a pressure of 80 and 800 bar with four circulations of the emulsion at room temperature using a 2 stage homogenizer (GEA Niro Soavi Spa, Parma, Italy). Ascorbic acid, ascorbyl palmitate or ascorbyl CLA was added directly to the continuous phase (buffer), whereas CLA was dissolved in methanol and then added to the buffer (0.5 mL). The same amount of methanol was added to all the other emulsions. The emulsions (100 g) were stored in 100 mL blue cap bottles on stirring plates in the dark (37 °C). One bottle per emulsion was taken on days 0, 4, 8, 12 and 15 and divided into brown glass bottles and stored at −40 °C until analyses of peroxides and volatiles were performed. The droplet size was measured on days 1, 8 and 15 without prefreezing. For the o/w emulsions, two references without antioxidants were included and the average of these two samples was used as a reference.

Droplet Size

*W/O Emulsions*

Droplet sizes were measured by a Zetasizer Nano-25 (Malvern Instruments Ltd., Worcestershire, UK). The emulsion was mixed with hexadecane (1:2 v/v). The following settings were used; refractive index (RI) for the oil was 1.434, viscosity of the disperse phase 5.5 mPa s and $RI_{water}$ was 1.330. The droplet size was calculated from

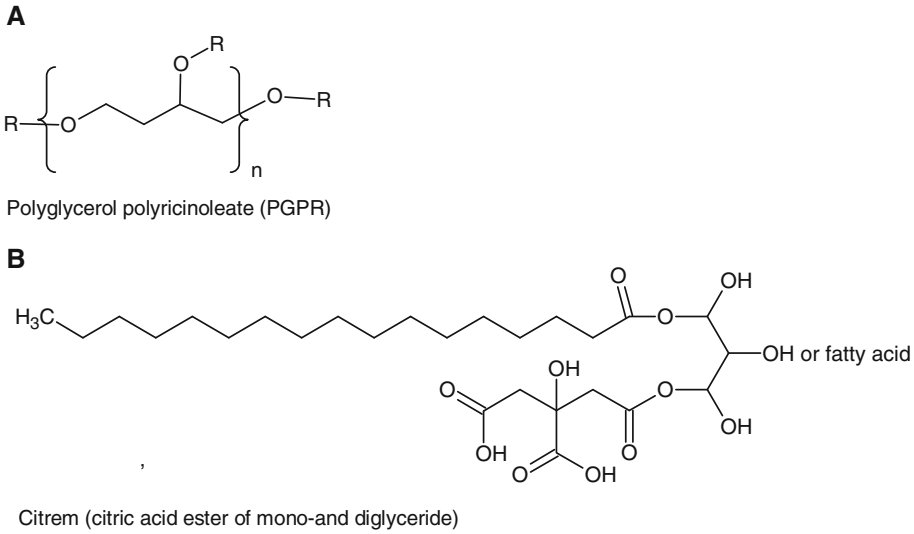


**Fig. 1** Structure of the two emulsifiers applied in two different emulsions system: w/o and o/w. **a** Polyglycerol polyricinoleate (PGPR), R = H or a fatty acyl group, $n$ = degree of polymerization of glycerol (average > 1). **b** Citrem (citric acid ester of mono- and diglyceride), citric acid ester of monoglyceride (-OH) and diglyceride (fatty acid)

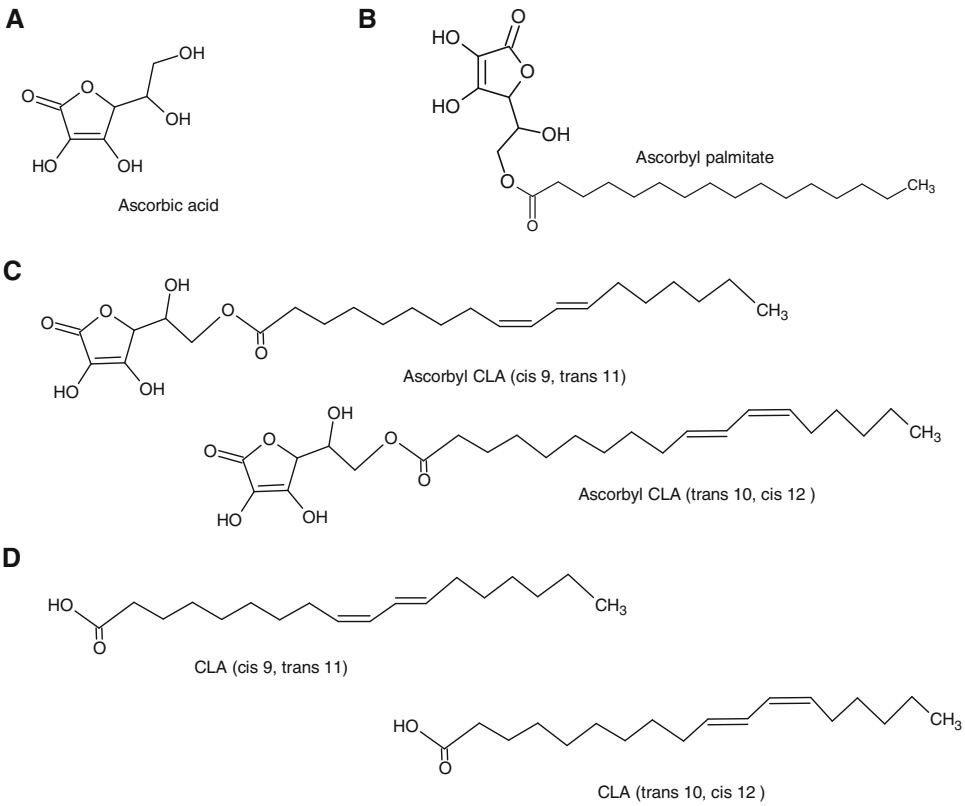


**Fig. 2** The structure of the compounds evaluated as antioxidants: **a** Ascorbic acid, **b** Ascorbyl palmitate, **c** Ascorbyl CLA and **d** CLA

viscosity of the continuous phase and the measured back scattering from particle movements. Triplicate measurements were performed.

*O/W Emulsions*

The size of the lipid droplets in the o/w emulsion was determined by laser diffraction using a Mastersizer2000 (Malvern Instruments Ltd., Worcestershire, UK). The o/w emulsion was diluted directly in recirculating water (3,000 rpm) reaching an obscuration of 12–14%. Sunflower oil ($RI_{oil} = 1.469$) and water ($RI_{water} = 1.330$) were used as particle and dispersant, respectively. Duplicate measurements were performed. Results are given in surface area mean diameter: $D_{3,2} = \frac{\sum d^3}{\sum d^2}$.

### Primary Oxidation Products, Peroxide Value (PV)

#### *W/O Emulsions*

Lipid hydroperoxides were extracted by a mixture of methanol and 1-butanol (2:1, v/v). After centrifugation for 3 min (2,000*g*), the clear upper layer (lipid phase) was used directly to quantify hydroperoxides by a modified method of Shanta and Decker [12]. Hydroperoxide concentrations were determined using a cumene hydroperoxide standard curve.

#### *O/W Emulsions*

Lipids were extracted from the emulsion according to a modified Bligh and Dyer method [13] using a reduced amount of methanol:chloroform, 1:1 v/v [14]. Hydroperoxides were measured as described for the lipid extracts from w/o emulsions, but the concentrations were calculated using a ferrichloride standard curve and a conversion factor of 2 to transform the unit from mequiv $O_2$ $kg^{-1}$ oil to mequiv peroxides $kg^{-1}$ oil. Duplicate measurements were performed.

### Secondary Oxidation Products

#### *W/O Emulsions*

Propanal and hexanal were determined directly in the stored GC vials using a GC 2014 Shimadzu gas chromatograph equipped with an autosampler (Shimadzu, Kyoto, Japan) and a DB-1 column (30 m × ID 0.32 mm × 1 mm film thickness, Supelco, Bellefonte, PA, USA). Each sample was heated at 55 °C in the autosampler heating block for 15 min and headspace volatiles were adsorbed on a solid phase microextraction (SPME) fiber needle (50/30 μm DVB/Carboxen/PDMS, Supelco, Bellefonte, PA) over 1 min. Volatiles were desorbed in the injector port at 250 °C for 3 min and passed through a DB-1 column in 10 min at 65 °C (FID temperature was 250 °C). Concentrations were determined from peak areas using propanal and hexanal standard curves prepared from propanal and hexanal in a w/o emulsion using MCT oil (medium chain triglyceride) in the emulsion. Results are given in ng $g^{-1}$ emulsion.

#### *O/W Emulsions*

Collection, separation and identification of volatiles was performed by dynamic headspace GC–MS (GC: 5890 IIA, Hewlett-Packard, CA, USA and MS: HP 5972 mass selective detector) equipped with a DB wax column (30 m x ID 0.25 mm × 0.5 μm film thickness, J&W Scientific, CA, USA). Volatiles from the emulsions (4 g) with 2–4 mL of Synperonic (antifoam) were purged from the emulsion (45 °C) with nitrogen for 30 min and trapped in Tenax tubes. The trapped volatiles were desorbed and analyzed on GC–MC. Settings for the GC temperature program were as follows: 45 °C for 4 min, 45–55 °C by increasing temperature at 1.5 °C/min, 55–90 °C by increasing the temperature at 2.5 °C/min, 90–220 °C by increasing the temperature at 12 °C/min and holding for 4 min. The temperature of the detector was 280 °C. For quantification of the different volatiles, solutions with external standards at different concentrations were prepared and analyzed directly in Tenax tubes. The analysis was performed in triplicate and results given in ng $g^{-1}$ emulsion.

### Tocopherol Concentration

#### *O/W Emulsion*

Approximately 3 g of the same lipid extract as used for PV analysis, was evaporated under nitrogen, re-dissolved in heptane and analyzed by HPLC (Agilent 1100 Series, Agilent Technology, CA, USA) according to the AOCS method [15] to determine tocopherol concentration in all emulsion samples. The reported tocopherol concentrations are averages of duplicate lipid extractions and duplicate analysis on the HPLC.

### Data Analysis

The results obtained were analyzed by two way analysis of variance (GraphPad Prism, Version 4.03, GraphPad Software, Inc). The Bonferroni multiple comparison was used to test differences between samples or storage time (significance level $p < 0.05$). When a significant difference was observed between two samples, they are denoted with different superscripts in the text, figure or table. To compare the efficacy of the antioxidants in the two different emulsion systems, inhibition percentages $\left[\text{Inhibition } (\%) = \left(1 - \frac{C_{\text{Volatile}_{\text{Sample,Antioxidant}}}}{C_{\text{Volatile}_{\text{Sample,Control}}}}\right) \times 100\right]$ were calculated.

## Results and Discussion

### Antioxidant Properties of the Compounds

Radical scavenging, iron chelation and reducing power activities were measured for ascorbyl palmitate, ascorbic acid, ascorbyl CLA and CLA.

The radical scavenging activity (Fig. 3a), measured by the DPPH assay, indicates whether the compound is able to

act as a free radical scavenger. It is very clear that CLA has no scavenging effect at all, whereas ascorbic acid, ascorbyl palmitate and ascorbyl CLA showed significantly higher scavenging effects than BHT (positive control). Their effects were concentration dependent and they had equal scavenging effects at each concentration.

Reducing power indicates the ability of a compound to donate electrons to reactive free radicals. Similar to the radical scavenging activity, CLA did not exert any reducing power (Fig. 3b). A concentration dependent effect was observed for ascorbic acid, ascorbyl CLA and ascorbyl palmitate, where higher concentrations resulted in higher reducing power. High reducing power was observed for ascorbic acid, while for ascorbyl palmitate and ascorbyl CLA it was very low. All three antioxidants had significantly different reducing power, and the ranking was as follows: ascorbic acid$^{d}$ > ascorbyl palmitate$^{c}$ > ascorbyl CLA$^{b}$ > CLA$^{a}$.

EDTA was used as positive control in the metal chelating activity assay, and showed a concentration dependent chelation between 50 and 100% in the tested concentration area. EDTA was observed to bind 50% ferrous ion at 25 μmol $L^{-1}$ and 99.7% was bound at both 102 and 205 μmol $L^{-1}$ EDTA concentrations (data not shown). The results showed that CLA had very limited chelation between 4 and 7% and for the remaining compounds the chelation was observed to be 0% using this assay (data not shown). Thus, the results indicate that the chelating activity was not as strong as ferrozine for these compounds.

Ascorbic acid is known to have multifunctional effects as a radical scavenger of hydrophilic radicals and reducing ability due to its ability to donate an electron to reactive free radicals. However, reducing power can result in prooxidative effects, if $Fe^{3+}$ is reduced to $Fe^{2+}$, especially in the presence of already existing lipid hydroperoxides [16]. In addition, ascorbic acid is known to act as a synergist with tocopherol by regenerating tocopherol from the tocopheroxyl radicals, formed after reaction between tocopherol and lipophilic radicals [16]. Due to the molecular structure of ascorbyl palmitate and ascorbyl CLA (Fig. 2), their antioxidative properties most likely rely on the ascorbyl group in these molecules.

The measured radical scavenging activities for ascorbic acid and its esters were similar to values earlier reported for ascorbic acid by Yen et al. [17]. Thus, the radical scavenging activity seemed independent of the esterification of ascorbic acid. In contrast, the reducing power was reduced by esterification of ascorbic acid (ascorbic acid > ascorbyl palmitate > ascorbyl CLA). These data indicate that the OH-group, which is changed by esterification, plays a role in the reducing power of ascorbic acid. This is in accordance with findings obtained with dihydrocaffeic acid esters by Sabally et al. [18], who suggested that the acid group in dihydrocaffeic acid contributed in the donation of H-atoms. The properties of ascorbic acid and ascorbyl palmitate were in accordance with earlier reports, which also observed radical scavenging activity and reducing properties of ascorbic acid and ascorbyl palmitate [19]. Moreover, the significant difference between ascorbyl palmitate and ascorbyl CLA might indicate that the degree of saturation and perhaps the chain length of the fatty acid had an impact on the reducing power.

The finding that CLA had no antioxidative properties except for very limited metal chelating activity conflicts with results obtained by Ha et al. [5] and Yu [20], but confirms with results obtained by van den Berg et al. [7]. The antioxidative properties of CLA reported in the study by Ha et al. [5] were based on PV measurements in an ethanol-buffer system containing linoleic acid, but the mechanism for the antioxidative properties of CLA was not explained. Moreover, Yu [20] showed radical scavenging activity of CLA at concentrations between 5 and 80 mg $L^{-1}$ and in accordance with our study, ascorbic acid was shown to be more efficient than CLA [20]. In the study by van den Berg et al. [7] neither radical scavenging nor metal chelating activity of CLA in radical or iron initiated

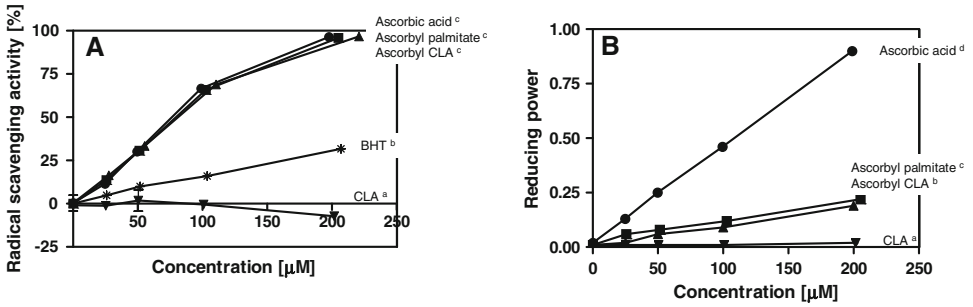


**Fig. 3** Antioxidant activity of ascorbic acid, ascorbyl palmitate, ascorbyl CLA and CLA. **a** Radical scavenging activity (*asterisks* indicates BHT included as a positive control) and **b** Reducing power. Legends: *filled circles* ascorbic acid, *filled squares* ascorbyl palmitate, *filled triangles* ascorbyl CLA and *filled inverted triangles* CLA, the *error bars* indicate the standard deviation of three measurements. Different *superscripts* after sample code indicate significant differences in the concentration range of 25–200 μmol $L^{-1}$

lipid oxidation were observed. It is unclear why CLA had limited chelating activity in the present study, whereas ascorbyl CLA showed no activity. Either, the activity can be ascribed to the acid group or it might be due to solubility problems, since the assay is normally conducted with compounds solubilized in water. However, CLA was not soluble in water and therefore it was solubilized in small amounts of methanol and then diluted with water. The result obtained by van den Berg et al. [7] and the molecular structure of CLA together suggest that the limited metal chelating properties of CLA observed in the present study was most likely due to interaction with methanol used for solubilization of CLA in our experiment.

## Physical Stability of Emulsions

The average droplet diameter of the aqueous droplets in the oil phase (w/o emulsion) was measured to 240–395 nm (Table 1). The droplet size in all these emulsions increased significantly from day 1 to day 4 or 8, thereafter the emulsion droplets did not change significantly (Table 1). However, the droplet size for the reference emulsion decreased from day 8 to 16, but this decrease in size cannot be explained by the present data. An exception was the emulsion with ascorbic acid for which the droplet size was stable through out the storage period. Thus, the w/o emulsions seemed to be physically unstable during storage, except for the emulsion with ascorbic acid added. However, no creaming was observed in the emulsions.

In contrast to the w/o emulsions, droplets in the o/w emulsions were stable during the entire storage period (data not shown). Average diameter of the oil droplets in the o/w emulsion was around 110 nm. The emulsion with ascorbic acid had slightly smaller droplets compared to the other emulsions.

## Partitioning of Compounds

All three compounds, ascorbic acid, ascorbyl palmitate and ascorbyl CLA, were detected in the aqueous buffer phase in the oil-buffer system. The concentration in the aqueous phase was highest for ascorbic acid followed by ascorbyl CLA and ascorbyl palmitate (Table 2). However, in the other two systems, buffer-emulsifier and emulsion, only ascorbic acid was detected in the aqueous phase and no ascorbyl palmitate or ascorbyl CLA could be detected in the aqueous phase (Table 2). This may partly be explained by the interaction of ascorbyl palmitate and ascorbyl CLA with surfactant.

The smaller amount of ascorbic acid detected in the aqueous phase in the buffer-emulsifier system compared with the oil-buffer system could indicate an interaction with citrem. However, in the emulsion system all ascorbic acid was detected in the aqueous phase, which indicates no interactions between ascorbic acid and citrem in this system. Moreover, at pH 7 both citrem and ascorbic acid is expected to be negatively charged, hence repulsive forces existed between the interface and ascorbic acid [21]. Thus, it might be that ascorbic acid was located away from the interface due to repulsive forces. In contrast, the emulsifier in the w/o emulsion, PGPR, is a non-ionic emulsifier, which enables ascorbic acid to be close to the interface in this emulsion system.

The partitioning data for the o/w system showed that in the buffer-oil system a little less than half of the amount of added antioxidant was detected in the buffer phase. However, when emulsifier (citrem) was present ascorbyl palmitate and ascorbyl CLA were not detected in the aqueous phase. This might indicate that ascorbyl CLA and ascorbyl palmitate partly interacted with citrem located at the interface or participated in micelles formed with citrem in the aqueous phase, but also that some ascorbyl CLA and ascorbyl palmitate partitioned into the oil phase. The critical micelle concentration (cmc) of citrem ($\approx$15 mg $L^{-1}$ [22]) is higher than the concentration of citrem (10 mg $L^{-1}$) in our experiment, hence it might generally be assumed that citrem is mainly located at the interface.

## Lipid Oxidation in Emulsions

The primary oxidation products, lipid hydroperoxides, were measured in both emulsion systems. Headspace

**Table 1** Droplet size of aqueous droplets (nm) measured after storage

| Droplet size, diameter (nm) | Day 1 | Day 4 | Day 8 | Day 16 |
|---|---|---|---|---|
| Reference | $265 \pm 7^{a}$ | $341 \pm 8^{bc}$ | $355 \pm 23^{c}$ | $297 \pm 24^{ab}$ |
| Ascorbic acid | $254 \pm 1^{a}$ | $291 \pm 19^{a}$ | $304 \pm 13^{a}$ | $281 \pm 21^{a}$ |
| Ascorbyl palmitate | $293 \pm 12^{a}$ | $327 \pm 5^{ab}$ | $395 \pm 54^{c}$ | $349 \pm 54^{bc}$ |
| Ascorbyl CLA | $260 \pm 5^{a}$ | $317 \pm 11^{b}$ | $304 \pm 18^{ab}$ | $300 \pm 17^{ab}$ |
| CLA | $241 \pm 1^{a}$ | $303 \pm 23^{b}$ | $345 \pm 47^{b}$ | $329 \pm 10^{b}$ |

Different superscripts in the same row indicate that droplet size changed significantly during storage for that specific emulsion. Standard deviation is based on three measurements ($n = 3$)

**Table 2** Concentration (%) of antioxidant measured in the aqueous phase (aq)

| Antioxidants | Oil/buffer (% in aq) | Citrem/buffer (% in aq) | Emulsion (o/w) (% in aq) |
|---|---|---|---|
| Ascorbic acid | 87 ± 10 | 70 ± 7 | 96 ± 1 |
| Ascorbyl palmitate | 48 ± 3 | – | – |
| Ascorbyl CLA | 57 ± 2 | – | – |

– indicates that the compound was not detected in the aqueous phase in the particular system

propanal and hexanal were determined in the w/o emulsions, whereas 1-penten-3-one, 2(t)-pentenal, 1-penten-3-ol, hexanal, 2(t)-hexenal, 4(c)-heptenal, nonanal, 2(t)-octenal and 2,4(t,t)-heptadienal were determined in the o/w emulsions.

For the w/o emulsion a clear lag phase was observed for lipid hydroperoxide concentrations in all samples (Fig. 4a). The lag phase was shortest for the reference emulsion (no antioxidant added) followed by CLA and ascorbic acid and the longest lag phase was observed for emulsions with ascorbyl CLA or ascorbyl palmitate added. Similarly, in the middle of the storage period, the reference had the highest concentration of lipid hydroperoxides followed by CLA and ascorbic acid and lowest concentration was observed in the emulsions with ascorbyl CLA and ascorbyl palmitate. During the entire storage period there was a lower concentration of lipid hydroperoxides in the emulsions with antioxidants compared to the reference emulsion.

Similar to the lipid hydroperoxides, a lag phase for headspace propanal was observed (Fig. 4b). The ranking was the same as for the lipid hydroperoxides: reference (shortest lag phase) < CLA = ascorbic acid = ascorbyl CLA = ascorbyl palmitate. All the compounds acted as antioxidants throughout the storage period. However, at the end of the storage period the efficacy of the compounds in retarding lipid oxidation was reduced as shown in Table 3. The concentration of propanal at day 16 was as follows in the different w/o emulsions: ascorbyl palmitate$^{a}$ = ascorbyl CLA$^{a}$ < ascorbic acid$^{b}$ ≤ CLA$^{b}$ < reference$^{c}$ (different superscripts indicate significantly different concentrations). The reduced efficacy of the applied compounds towards the end of the storage period might indicate depletion of them.

For the concentrations of hexanal, no significant differences between the efficacies of the different compounds were observed in the beginning and middle of the storage period (Table 3). At the end of the storage period the hexanal concentration in the emulsion was as follows: ascorbic acid$^{a}$ = ascorbyl CLA$^{a}$ = ascorbyl palmitate$^{a}$ ≤ CLA$^{ab}$ ≤ reference$^{b}$ (highest concentration), data not shown. A tendency that CLA resulted in a higher concentration of hexanal than the other antioxidants was

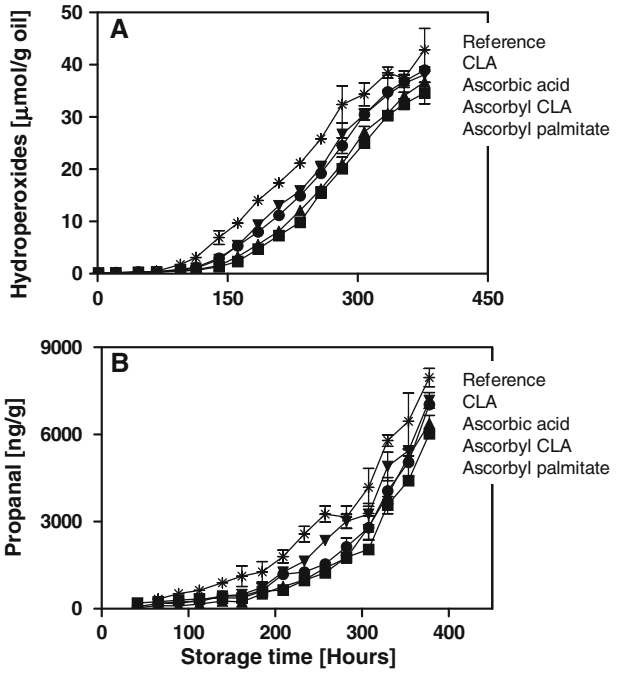


**Fig. 4** Concentration of lipid hydroperoxides (µmol $g^{-1}$ oil) (**a**) and propanal (ng $g^{-1}$) (**b**) in w/o emulsions during storage (h). *Asterisks* reference, *filled circles* ascorbic acid, *filled squares* ascorbyl palmitate, *filled triangles* ascorbyl CLA and *inverted filled triangles* CLA, the *error bars* indicate the standard deviation of three measurements

observed, however, the hexanal concentration in the CLA emulsion was not significantly different from the reference.

In contrast to the findings for w/o emulsions, no lag phase was observed for lipid hydroperoxides in the o/w emulsions (Table 4). However, there was a lower concentration of lipid hydroperoxides at day 4 in emulsions with ascorbyl CLA, ascorbic acid and ascorbyl palmitate added compared to the reference emulsion (no antioxidant added). The pattern in the PV data became more complex. For CLA and ascorbic acid emulsions, PV continued to increase and these two emulsions had the highest PV after 15 days. In contrast, PV in emulsions with ascorbyl palmitate, ascorbyl CLA or without antioxidants decreased towards the end of the storage period. After 15 days, the reference and the ascorbyl CLA emulsions had the lowest PV.

The development of the different volatiles was significantly affected in o/w emulsions by the antioxidant addition as shown by inhibition percentages of the applied antioxidants compared with reference emulsion (Table 3). No lag phase for any of the measured volatiles was observed (Table 4). In the beginning of the storage period ascorbic acid and ascorbyl palmitate reduced the formation of hexanal, 1-penten-3-one, 1-penten-3-ol and 4-heptenal and had no effect on the development of 2-hexenal and 2-octenal (Table 3, Table 4). In contrast, ascorbyl CLA and CLA had no or prooxidative effect on all volatiles. At the

**Table 3** Inhibition percentages of the development of volatiles in w/o and o/w emulsions during storage depended on the compound added

| Volatiles | Storage (days) | AA (%) | AP (%) | ACLA (%) | CLA (%) |
|---|---|---|---|---|---|
| W/O emulsion | | | | | |
| Propanal | 5 | 60 | 46 | 75 | 53 |
| | 12 | 32 | 44 | 45 | 5 |
| | 15 | 12 | 24 | 20 | 10 |
| Hexanal | 5 | 86 | 85 | 76 | 66 |
| | 12 | 16 | 24 | 48 | −57 |
| | 15 | 41 | 34 | 35 | 26 |
| O/W emulsion | | | | | |
| Hexanal | 4 | 25 | 100 | −46 | −2 |
| | 8 | 4 | 26 | −9 | 25 |
| | 16 | −172 | −212 | −57 | −222 |
| 1-penten-3-one | 4 | 64 | 90 | 23 | −28 |
| | 8 | 7 | 24 | −10 | −3 |
| | 16 | −209 | −239 | 30 | −188 |
| 2-pentenal | 4 | 21 | 100 | 21 | −14 |
| | 8 | 30 | 53 | 17 | 20 |
| | 16 | −144 | −248 | −48 | −220 |
| 1-penten-3-ol | 4 | 62 | 70 | 37 | 38 |
| | 8 | 30 | −36 | −7 | 23 |
| | 16 | −69 | −118 | −5 | −83 |
| 2-hexenal | 4 | – | – | – | – |
| | 8 | – | – | – | – |
| | 16 | −62 | −131 | −46 | −77 |
| 4-heptenal | 4 | 100 | 100 | 100 | −80 |
| | 8 | 47 | 32 | 26 | 21 |
| | 16 | −25 | −67 | −8 | −42 |
| Nonanal | 4 | −3 | −91 | −73 | −6 |
| | 8 | 16 | −52 | −20 | 34 |
| | 16 | −66 | −168 | −52 | −70 |
| 2-octenal | 4 | −100 | 100 | −400 | 100 |
| | 8 | 100 | 13 | −50 | 25 |
| | 16 | −85 | −162 | −100 | −154 |
| 2,4-heptadienal | 4 | 68 | 89 | 12 | 15 |
| | 8 | 50 | 41 | 9 | 23 |
| | 16 | 30 | 15 | 6 | 30 |

– indicates that the amount was below the detection limit for both the emulsion with antioxidant and without antioxidant (reference), whereas 100 indicates that the concentration of volatiles in emulsion with antioxidants was below detection limit but not the reference emulsion. Negative values indicate prooxidative effect of the respective antioxidant that particular day

*AA* ascorbic acid, *AP* ascorbyl palmitate, *CLA* conjugated linoleic acid, *ACLA* ascorbyl CLA

end of the storage period all the compounds generally resulted in a significantly increased formation of volatiles compared to the reference, except for 2,4-heptadienal (Table 4). For this compound the concentration was higher in the reference emulsion than in the emulsions with antioxidants throughout the storage period and at day 15 the ranking was as follows: ascorbic acid$^{a}$ = CLA$^{a}$ ≤ ascorbyl palmitate$^{ab}$ ≤ ascorbyl CLA$^{bc}$ ≤ reference$^{c}$. Interestingly, the data for ascorbyl CLA and ascorbyl palmitate showed that despite their similar molecular structures, they behaved differently. Ascorbyl palmitate was most efficient in reducing the development of volatiles in the beginning of the storage period, but at the end of the storage it was the most prooxidative compound tested seen from the concentration in this emulsion of most of the volatiles measured. The observation that antioxidants changed from being antioxidative at day 4 to being prooxidative at day 15 might indicate depletion of them during storage. A decrease in PV towards the end of the storage period in the o/w emulsion with ascorbyl palmitate, ascorbyl CLA or without antioxidants could indicate an increased formation of volatiles in these samples or formation of polymers. However, the increased formation of volatiles could only be confirmed for the emulsion with ascorbyl palmitate.

In addition to PV and volatiles, the tocopherol concentration in the different o/w emulsions was measured. Results showed that almost the same amount of α-tocopherol was consumed during storage in all emulsions. The consumption varied from 13 (ascorbyl CLA) to 15 μg kg$^{-1}$ (ascorbyl palmitate). In contrast, the amount of consumed γ-tocopherol depended upon the antioxidant added to the emulsion, and the ranking of the concentrations was as follows at the end of the storage period: reference = ascorbyl CLA > ascorbyl palmitate = ascorbic acid = CLA (Fig. 5). This ranking could indicate that the reference and the ascorbyl CLA emulsions oxidized less than the other emulsions. This finding was in accordance with PV data and most of the volatiles data at day 15, which also showed that ascorbyl CLA and the reference were less oxidized in the end of the storage.

In summary our results showed that in the w/o emulsion all four compounds acted as antioxidants and for lipid hydroperoxide and propanal measurements their activity was best in the middle of the storage period. The best antioxidative activity was observed for ascorbyl palmitate and ascorbyl CLA. In the o/w emulsion all 4 compounds had different effects on the development of the different volatiles. Generally, ascorbic acid and ascorbyl palmitate were most efficient in the beginning of the storage period. However, all the tested compounds acted as prooxidants at the end of the storage period, although ascorbyl CLA was less prooxidative than the other antioxidants.

## Lipid Oxidation in W/O Versus O/W Emulsions

The results clearly showed that lipid oxidation was initiated much faster in the o/w emulsion than the w/o emulsion. To

**Table 4** Concentration (ng $g^{-1}$ emulsion) of primary (PV) and two secondary (1-penten-3-one and 2,4-heptadienal) oxidation products in o/w emulsions measured at different time points (days)

| | Storage time (days) | | | | |
|---|---|---|---|---|---|
| | 0 | 4 | 9 | 12 | 15 |
| PV (mequiv peroxides/kg oil) | | | | | |
| Reference | 0.2 ± 0.05$^{a}$ | 27.8 ± 1.60$^{c}$ | 19.8 ± 2.74$^{a}$ | 23.0 ± 10.5$^{b}$ | 8.6 ± 1.24$^{a}$ |
| Ascorbic acid | 0.1 ± 0.03$^{a}$ | 16.5 ± 0.09$^{ab}$ | 33.2 ± 0.98$^{c}$ | 22.0 ± 0.56$^{b}$ | 36.5 ± 0.20$^{c}$ |
| Ascorbyl palmitate | 0.0 ± 0.04$^{a}$ | 10.0 ± 0.05$^{a}$ | 28.4 ± 0.41$^{bc}$ | 11.0 ± 0.09$^{a}$ | 22.6 ± 0.19$^{b}$ |
| Ascorbyl CLA | 0.0 ± 0.00$^{a}$ | 23.8 ± 0.25$^{bc}$ | 22.2 ± 1.20$^{ab}$ | 6.1 ± 0.06$^{a}$ | 6.3 ± 0.10$^{a}$ |
| CLA | 0.0 ± 0.30$^{a}$ | 27.0 ± 0.20$^{c}$ | 28.1 ± 1.27$^{bc}$ | 34.5 ± 0.23$^{c}$ | 37.1 ± 0.31$^{c}$ |
| 1-penten-3-one (ng/g emulsion) | | | | | |
| Reference | 0 | 64.0 ± 27.7 $^{cd}$ | 90.6 ± 22.3$^{ab}$ | 93.9 ± 24.6 $^{cd}$ | 32.9 ± 6.3$^{a}$ |
| Ascorbic acid | 0 | 23.0 ± 1.1$^{ab}$ | 85.4 ± 12.0$^{ab}$ | 74.4 ± 3.0$^{bc}$ | 102 ± 12.0$^{b}$ |
| Ascorbyl palmitate | 0 | 6.1 ± 0.5$^{a}$ | 69.0 ± 5.8$^{a}$ | 60.4 ± 7.7$^{ab}$ | 112 ± 8.7$^{b}$ |
| Ascorbyl CLA | 0 | 48.5 ± 6.7$^{bc}$ | 99.3 ± 7.8$^{b}$ | 40.8 ± 8.3$^{a}$ | 23.7 ± 5.2$^{a}$ |
| CLA | 0 | 82.1 ± 4.4$^{d}$ | 93.8 ± 7.0$^{ab}$ | 107 ± 5.9$^{d}$ | 95.4 ± 2.4$^{b}$ |
| 2,4-heptadienal (ng/g emulsion) | | | | | |
| Reference | 0 | 1,646 ± 5$^{c}$ | 4,469 ± 549$^{d}$ | 4,588 ± 1592$^{b}$ | 5,019 ± 282$^{b}$ |
| Ascorbic acid | 0 | 524 ± 54$^{ab}$ | 2,256 ± 297$^{a}$ | 4,305 ± 130$^{ab}$ | 3,517 ± 127$^{a}$ |
| Ascorbyl palmitate | 0 | 190 ± 27$^{a}$ | 2,653 ± 310$^{ab}$ | 5,296 ± 292$^{bc}$ | 4,255 ± 156$^{ab}$ |
| Ascorbyl CLA | 0 | 1,454 ± 29$^{bc}$ | 4,057 ± 401$^{cd}$ | 5,790 ± 86$^{c}$ | 4,713 ± 88$^{b}$ |
| CLA | 0 | 1,406 ± 437$^{bc}$ | 3,449 ± 261$^{bc}$ | 3,558 ± 165$^{a}$ | 3,521 ± 256$^{a}$ |

Different superscripts in a column within the same oxidation product indicate significant different concentrations in the two emulsions

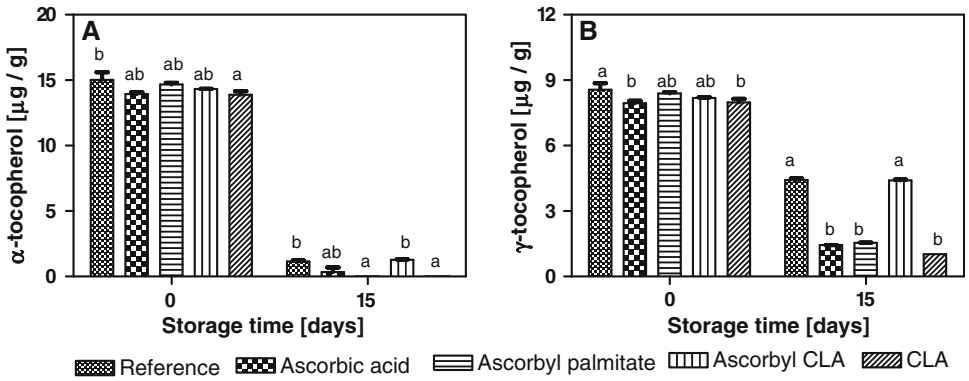


**Fig. 5** Concentration of **a** α- and **b** γ-tocopherol (µg $g^{-1}$) in o/w emulsions measured at day 0 and 15. The *error bars* indicate the standard deviation of four measurements for emulsion with antioxidants and eight measurements for the reference emulsion. Different *subscriptions* at the same day indicate a significant difference in the concentrations between these emulsions

the best of our knowledge, comparison of lipid oxidation in o/w versus w/o emulsion has not previously been reported. Instead, lipid oxidation rates for bulk oil and o/w emulsions have been compared [1, 23, 24]. All these studies indicated, that lipid oxidation was faster in o/w emulsions. In this study, the w/o emulsion contained only 1% water and 1% emulsifier and it thus resembles bulk oil to some extent as bulk oil also contains small amounts of water and surface active compounds such as free fatty acid, monoacylglycerols and diacylglycerols [25].

The different lag phases in the two systems might be due to the differences in droplet size i.e. total surface area, as the initial step in lipid oxidation takes place at the interface [9]. In the present study, the droplet size was smallest and the total surface area thus largest in the o/w emulsions. Some studies on emulsions support this interpretation of the data [26], whereas other studies have found the opposite. In studies with fish oil enriched milk, lipid oxidation decreased with decreasing droplet sizes [27]. These findings were explained by a more favorable protein composition at the interface in emulsions with small droplets. A similar change in the composition of the interface most likely did not take place in the present study due to the much simpler emulsifier system applied. However, the interfaces between the droplets and continuous phase in the o/w emulsion with citrem as the emulsifier, are negatively charged and thus attract metals. Therefore, the reason for the faster lipid oxidation in o/w emulsion could be due to

the larger surface and negatively charged droplets in this emulsion system compared to the w/o emulsion.

In addition, factors such as different emulsifiers, production processes and especially storage conditions might also be a reason for the different oxidative stability observed for the o/w versus w/o emulsions. The o/w emulsions were continuously stirred during storage, which means that headspace oxygen was distributed in the emulsion. This is suggested as being an important reason for the faster initiation of oxidation in the o/w emulsion.

## Antioxidant Effects in W/O versus O/W Emulsions

The efficacy of the different compounds tested as antioxidants in the two emulsions systems was different. Both systems were multiphase systems and according to the polar paradox hypothesis, amphiphilic antioxidants are more efficient compared to hydrophilic and lipophilic antioxidants in protecting against lipid oxidation in emulsions [1, 2]. In this study, the polar paradox hypothesis was partly confirmed, since ascorbyl palmitate was a better antioxidant than ascorbic acid at the beginning of the storage period in the o/w emulsions. However, ascorbyl CLA was practically inactive, and at the end of the storage period all antioxidants seemed to promote formation of most of the measured volatile oxidation products. Moreover, ascorbyl palmitate became more prooxidative than ascorbic acid towards the end of the storage period. Earlier studies also showed antioxidant effects that differed from the polar paradox [28], and it was concluded that the polar paradox was too simple to explain antioxidant effects in multiphase systems as emulsions, most likely because of interactions between iron, emulsifiers and antioxidants [28].

The ascorbyl CLA was not a completely purified product after esterification and contained free fatty acids, which can have a prooxidative effect in o/w emulsions together with iron [29]. However, this might be more important in the w/o emulsions, where the droplets were not negatively charged. Moreover, iron was not added to the emulsions in this study, however it is expected to be present in the oil in trace amounts. Thus, the antioxidative activity of ascorbyl CLA may be neutralized due to its content of free fatty acids and this could explain its poor effect in the o/w emulsion. Furthermore, different chain lengths of ascorbic acid esters have been shown to influence their efficacy as antioxidant in copper and metal initiated LDL oxidation as follows: ascorbyl laurate > ascorbyl palmitate > ascorbyl caprylate [8]. Thus, the chain length and saturation may influence their efficacy as antioxidant.

In the w/o emulsions ascorbyl palmitate and ascorbyl CLA were more efficient than ascorbic acid and CLA, which is in accordance with the polar paradox for emulsions. As described, the w/o emulsion resembled bulk oil to some extent although the structure of the two systems is different. In bulk oil, water is located in micelles, whereas in w/o emulsions the aqueous phase is surrounded by emulsifier. Our findings may therefore indicate that the type of interface has an influence. In addition, CLA acted as an antioxidant in the w/o emulsion, whereas in the o/w emulsion CLA was inactive or had prooxidative effect on the development of the different volatiles.

Previously we hypothesized that hydrophilic antioxidants might offer better protection in a w/o emulsion compared to the reverse o/w emulsions if the concentration of the disperse phase was equal. In our experiment the amount of disperse phase was less in the w/o emulsions (1%) than in the o/w emulsions (5%). Accordingly, it was observed that ascorbic acid inhibited oxidation in the w/o emulsion more than in the o/w emulsion. We suggest that ascorbic acid is closer to the interface in a w/o emulsion due to its higher concentration in the aqueous phase compared to an o/w emulsion and therefore exhibits higher antioxidative efficacy. In addition, the concentration of the disperse phase in our w/o emulsion was smaller than the disperse phase in our o/w emulsion with even higher concentration of ascorbic acid in the aqueous phase of the w/o emulsions; 10,000 versus 106 μmol $L^{-1}$ in the aqueous phase in w/o and o/w emulsions, respectively. However, interactions with other compounds such as emulsifier might also be considered [29].

## Partitioning of the Antioxidants Related to Their Efficacy

The negative charge of citrem and neutral charge of PGPR, might also be a reason for the higher efficiency of ascorbic acid in the w/o emulsions. However, partitioning studies with PGPR and ascorbic acid are required to conclude further.

The structure of ascorbyl palmitate and ascorbyl CLA only differs in the fatty acyl group by the chain length and degree of saturation (Fig. 2). Hence, it might be speculated that the structural differences result in different interactions between citrem and ascorbyl CLA than between citrem and ascorbyl palmitate, which reduces the possibility of ascorbyl CLA to reduce lipid oxidation. After all, the data from the oil/buffer system indicated a higher concentration of ascorbyl palmitate in the oil phase compared to ascorbyl CLA. This may partly explain why it was more efficient than ascorbyl CLA in the beginning of the storage period.

Although ascorbyl CLA was located closer to the lipids than ascorbic acid and therefore supposed to be more efficient than ascorbic acid, it was ascorbic acid that was more efficient as an antioxidant compared to ascorbyl CLA in o/w emulsion in the beginning of the storage. However,

both ascorbyl CLA and ascorbyl palmitate had less reducing and radical scavenging activity compared to ascorbic acid. Ascorbic acid was suggested to be repelled from the interface by citrem, however the repulsive forces may not be that strong.

For further specification of the mechanism that results in different antioxidative action of ascorbyl CLA and ascorbyl palmitate and better protection with ascorbic acid than ascorbyl CLA in o/w emulsions, studies on interaction of these compounds with citrem are required.

## Conclusion

Initiation of lipid oxidation was influenced by the emulsion system. Oxidation occurred faster in the o/w emulsions compared to w/o emulsions and this was suggested to be due to smaller droplets and continuous stirring during storage of the o/w emulsions. As hypothesized, ascorbic acid was more efficient in preventing the initial lipid oxidation in the w/o emulsions compared to o/w emulsions. This is explained by the higher concentration of ascorbic acid in the aqueous phase in w/o emulsions and hence a closer location to the interface. However, the polar paradox hypothesis was only partly confirmed. In the w/o emulsion system the amphiphilic antioxidants, ascorbyl palmitate and ascorbyl CLA, were more efficient than the hydrophilic antioxidant, ascorbic acid.

The efficacy of the antioxidants in the o/w emulsions was more complex, since ascorbyl palmitate and ascorbic acid were most efficient at the beginning of the storage and towards the end they acted as prooxidants. In addition, ascorbyl CLA was practically inactive. Since ascorbyl CLA was located at the interface or in the oil phase in o/w emulsions and thus was supposed to work efficiently as an antioxidant, the inactivity of this compound as an antioxidant may be due to impurities such as free fatty acids or an interaction with citrem that prevent it from acting as antioxidant.

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## References

1. Frankel EN, Huang S-W, Kanner J, German JB (1994) Interfacial phenomena in the evaluation of antioxidants: bulk oils vs emulsions. J Agric Food Chem 42:1054–1059
2. Porter WL (1993) Paradoxical behavior of antioxidants in food and biological systems. Toxicol Ind Health 9(1–2):93–122
3. Jacobsen C, Let MB, Nielsen NS, Meyer AS (2008) Antioxidant strategies for preventing oxidative flavour deterioration of foods enriched with n-3 polyunsaturated lipids: a comparative evaluation. Trends Food Sci Technol 19(2):76–93
4. Ip C, Chin SF, Scimeca JA, Pariza MW (1991) Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. Cancer Res 51:6118–6124
5. Ha YL, Storkson J, Pariza MW (1990) Inhibition of Benzo(a)-pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. Cancer Res 50:1097–1101
6. MacDonald HB (2000) Conjugated linoleic acid and disease prevention: a review of current knowledge. J Am Coll Nutr 19(2):111S–118S
7. van den Berg JJM, Cook NE, Tribble DL (1995) Reinvestigation of the antioxidant properties of conjugated linoleic acid. Lipids 30:599–605
8. Liu Z-Q, Ma L-P, Liu Z-L (1998) Making vitamin C lipophilic enhances its protective effect against free radical induced peroxidation of low density lipoprotein. Chem Phys Lipids 95:49–57
9. McClements DJ, Decker EA (2000) Lipid oxidation in oil-in-water emulsions: impact of molecular environment on chemical reactions in heterogeneous food systems. J Food Sci 65(8):1270–1282
10. Frankel EN, Huang S-W, Prior E, Aeschbach R (1996) Evaluation of antioxidant activity of rosemary extracts, carnosol and carnosic acid in bulk vegetable oils and fish oil and their emulsions. J Sci Food Agric 72(2):201–208
11. Schwarz K, Frankel EN, German JB (1996) Partition behaviour of antioxidative phenolic compounds in heterophasic systems. Fett Lipid 98(3):115–121
12. Shantha NC, Decker EA (1994) Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. J AOAC Int 77(2):421–424
13. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917
14. Iverson SJ, Lang SLC, Cooper MH (2001) Comparison of the Bligh and Dyer and Folch methods for total lipid determination in broad range of marine tissue. Lipids 36(11):1283–1287
15. AOCS Official Method Ce 8–89 (1997) Determination of tocopherols, tocotrienols in vegetable oils, fats by HPLC. Champaign, IL
16. Niki E (1991) Action of ascorbic acid as a scavenger of active and stable oxygen radicals. Am J Clin Nutr 54:1119s–1124s
17. Yen GC, Duh PD, Tsai HL (2002) Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. Food Chem 79(3):307–313
18. Sabally K, Karboune S, St-Louis R, Kermasha S (2007) Lipase-catalyzed synthesis of phenolic lipids from fish liver oil and dihydrocaffeic acid. Biocatal Biotransformation 25(2–4):211–218
19. Mäkinen M, Kähkönen M, Hopia A (2001) Ascorbic acid and ascorbyl palmitate have only minor effect on the formation and decomposition of methyl linoleate hydroperoxides. Eur J Lipid Sci Technol 103:683–687
20. Yu L (2001) Free radical scavenging properties of conjugated linoleic acids. J Agric Food Chem 49:3452–3456
21. Frankel EN, Meyer AS (2000) The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. J Sci Food Agric 80(13):1925–1941
22. Semenova MG, Belyakova LE, Polikarpov YN, Stankovic I, Antipova AS, Anokhina MS (2007) Analysis of light scattering data on the sodium caseinate assembly as a response to the interactions with likely charged anionic surfactant. Food Hydrocoll 21(5–6):704–715
23. Frankel EN, Satué-Gracia T, Meyer AS, German JB (2002) Oxidative stability of fish and algae oils containing long-chain

polyunsaturated fatty acids in bulk and in oil-in-water emulsions. J Agric Food Chem 50:2094–2099
24. Huang S-W, Hopia A, Schwarz K, Frankel EN, German JB (1996) Antioxidant activity of α-tocopherol and trolox in different lipid substrates: bulk oils vs oil-in-water emulsions. J Agric Food Chem 44:444–452
25. Chaiyasit W, Elias RJ, McClements DJ, Decker EA (2007) Role of physical structures in bulk oils on lipid oxidation. Crit Rev Food Sci Nutr 47(3):299–317
26. Gohtani S, Sirendi M, Yamamoto N, Kajikawa K, Yamano Y (1999) Effect of droplet size on oxidation of docosahexaenoic acid in emulsion system. J Dispers Sci Technol 20(5):1319–1325
27. Sørensen A-DM, Baron CP, Let MB, Brüggemann DA, Pedersen LRL, Jacobsen C (2007) Homogenization conditions affect the oxidative stability of fish oil enriched milk emulsions: oxidation linked to changes in protein composition at the oil-water interface. J Agric Food Chem 55:1781–1789
28. Sørensen A-DM, Haahr A-M, Becker EM, Skibsted LH, Bergenståhl B, Nilsson L, Jacobsen C (2008) Interactions between iron, phenolic compounds, emulsifiers, and pH in omega-3-enriched oil-in-water emulsions. J Agric Food Chem 56:1740–1750
29. Waraho T, Cardenia V, Rodriguez-Estrada MT, McClements DJ, Decker EA (2009) Prooxidant mechanisms of free fatty acids in stripped soybean oil-in-water emulsions. J Agric Food Chem 57(15):7112–7117