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Complete List of Authors:	Sørensen, Ann-Dorit Moltke; Technical Univesity of Denmark, National Food Institute Villeneuve, Pierre; CIRAD, AMIS, UMR IATE Jacobsen, Charlotte; Technical University of Denmark, National Food Institute, Division of Industrial Food Research

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Alkyl caffeates as antioxidants in O/W emulsions: Impact of emulsifier type and endogenous tocopherols

Ann-Dorit Moltke Sørensen[1]*, Pierre Villeneuve[2], Charlotte Jacobsen[1]

[1] Division of Food Technology, National Food Institute (DTU Food), Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

[2] CIRAD, UMR IATE, Montpellier, F-34398, France

[*] Corresponding author: adms@food.dtu.dk, Phone: +45 4525 2591

Running title: Effect of emulsifier and antioxidants on lipid oxidation

Keywords: Caffeic acid; Lipid oxidation; Tween; Citrem; Phenolipids; Fish oil; Antioxidant interactions

Abbreviations: CA Caffeic acid; C1 Methyl caffeate; C4 Butyl caffeate; C8 Octyl caffeate; C12 Dodecyl caffeate; C16 Hexadecyl caffeate; C20 Eicosyl caffeate; CAT Conjugated autoxidizable triene; CCL Critical chain length; FO Fish oil; FO/RO Fish oil and rapeseed oil mixture (1:1); PC Principal component; PCA Principal component analysis; PV Peroxide value; THF Tetrahydrofuran

Abstract

Antioxidant addition can be one strategy to limit lipid oxidation in emulsions. Research has proven that an important factor regarding the efficacy of antioxidants is their localization in the emulsion; however, other factors such as interactions with other components can also have an impact. Thus, the aim was to evaluate the impact of emulsifiers (Citrem and Tween80) and presence of endogenous tocopherols on the efficacies of caffeic acid and caffeates (C1-C20) as antioxidants in emulsions. Lipid oxidation was evaluated during storage and partitioning of caffeic acid and caffeates was estimated by measuring their concentrations in the aqueous phase.

Partitioning of caffeic acid and caffeates was influenced by emulsifier type and presence of endogenous tocopherols. Caffeic acid was the most efficient antioxidant in Citrem and Tween stabilized emulsions in the presence of endogenous tocopherol. In contrast, for Tween stabilized emulsions, caffeic acid acted as a prooxidant and the evaluated caffeates acted as strong antioxidants in the absence of endogenous tocopherol. Thus, when endogenous tocopherol was present lipophilization of caffeic acid did not increase its efficacy as an antioxidant. It is suggested that the differences observed in antioxidant efficiency with different emulsifiers and with and without endogenous tocopherols is due to emulsifier-antioxidant interactions and antioxidant-antioxidant interactions in the emulsions.

Practical application: Food emulsions contain endogenous tocopherols, thus, the impact of endogenous tocopherols on the efficacy of applied antioxidants is of interest to the industry. So far the hypotheses about antioxidant in emulsions are based on simple emulsions systems without the presence of tocopherols. The finding in this study revealed that both emulsifier type and the presence of endogenous tocopherol had an impact on the efficacy of caffeic acid and caffeates due to emulsifier-antioxidant and antioxidant-antioxidant interactions. This highlights the importance of

evaluating the antioxidant in each emulsion system before selecting antioxidants for optimal protection against lipid oxidation.

Introduction

An o/w emulsion consists of three different phases. A dispersed phase (oil) is present as droplets in a continuous phase (water) and separated by an interfacial region. Type of emulsions can range from very simple, when prepared from only a few ingredients, to more complex, when prepared with many different ingredients e.g. food emulsions. Lipid oxidation can occur rapidly in emulsions due to their large interfacial area. The interface region facilitates interactions between the lipids and water-soluble prooxidants [1].

Different strategies can be applied to limit lipid oxidation and thereby improve shelf life of emulsions [2]. One of them is addition of antioxidants; however, selection of the right antioxidant or mixture of antioxidants is difficult, since their efficacies are affected by the composition of the emulsions e.g. their localization and interaction with other components. An enormous amount of studies have been reported in the literature on antioxidants and their efficacies in model emulsions and more complex food emulsions. So far, two hypotheses about antioxidant efficacies in emulsions, namely the polar paradox hypothesis [3] and the cut-off effect [4] have been reported. In brief, the polar paradox hypothesizes that apolar antioxidants are more efficient in O/W emulsions than polar antioxidants [3] due to differences in the antioxidants' affinity towards the different phases [5]. The cut-off effect can be seen as an extension of the polar paradox hypothesis, in which an optimal degree of lipophilisation for optimal antioxidant activity is observed [4], which is called critical chain length (CCL). The cut-off effect was observed from results obtained with chlorogenic acid and rosmarinic acid and their unbranched saturated alkyl esters (chlorogenates and rosmarinates). The efficacy of antioxidant homologues was related to the partitioning of these antioxidants in an emulsion system [4, 6]. Based on these observations, it was assumed that when the lipophilized antioxidants had the CCL they were present in the highest concentration at the oil-aqueous interface, where lipid oxidation is initiated. In addition, antioxidant homologues with chain length below and above CCL were driven away from the oil-aqueous interface [7]. These antioxidant hypotheses are based on extensive research in simplified emulsions prepared with stripped oils, whereas food emulsions contain e.g. endogenous tocopherols. Only few compounds with different degree of lipophilization have been evaluated in several different emulsion systems. Caffeic acid and unbranched saturated caffeates have been evaluated both in a model emulsion (CAT assay) system [8], milk and mayonnaise [9] and CCL seemed to be influenced by the system. In addition, rosmarinic acid has also been evaluated in different systems; model emulsions and low moisture food (crackers). In model emulsions, a parabolic relationship between antioxidant efficacy and hydrophobicity was observed, with the intermediate polarity (8 carbon – 18 carbon chain length) giving optimum activity [6, 10, 11]. In crackers, a linear relationship between antioxidant activity and hydrophobicity was observed [12].

Due to formerly obtained results with caffeic acid and caffeates in model emulsion, milk and mayonnaise [8, 9], it is hypothesized that emulsifier type and the presence of endogenous tocopherols can affect the partitioning of caffeic acid and caffeates and thereby change their efficacy in the emulsion systems. Hence, the aim of this study was to evaluate the impact of both emulsifier and presence of endogenous tocopherols on the efficacies of caffeic acid and caffeates (C1-C20). Two experiments were carried out as shown in Table 1. In the first experiment, the aim was to evaluate the effect of lipophilization of caffeic acid on its antioxidant efficacy in an O/W emulsion prepared with unstripped oil and Citrem as emulsifier. The aim of the second experiment was to investigate whether the presence of endogenous tocopherol affected the optimal chain length of lipophilized caffeic acid when Tween was used as an emulsifier. Moreover, comparison of results of the two experiments enabled an evaluation of whether the emulsifier type, Citrem vs Tween affected the CCL in emulsions prepared with unstripped oil.

In both experiments, the partitioning of the antioxidant was estimated by measuring its concentration in the aqueous phase of the O/W emulsion, a buffer/oil system and a buffer/emulsifier system to evaluate if emulsifier type and the presence of endogenous tocopherols, affected the partitioning of caffeic acid and caffeates in the emulsion system.

Material and Methods

The oil used in this study was fish oil (FO) and a mixture between fish and rapeseed oil (FO/RO, 1:1). The two types of oil were supplied by Maritex A/S (TINE BA, Sortland, Norway). The quality of the FO was as follows: 0.3 meq. peroxides / kg oil, 250 mg α-tocopherol / kg, 98 mg γ-tocopherol / kg and 48 mg δ-tocopherol / kg. Fatty acid composition of the fish oil 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-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, 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 the FO were 24.0% and 1.8 %, respectively. The quality of the FO/RO was as follows: 0.3 meq. peroxides / kg oil, 230 mg α-tocopherol / kg, 31 mg β-tocopherol / kg, 151 mg γ-tocopherol / kg and 6 mg δ-tocopherol / kg. The fatty acid composition of the FO/RO was as follows: 14:0, 1.7%; 16:0, 7.1%; 16:1 (n-7), 4.6%; 18:0, 1.9%; 18:1 (n-9), 38.3%; 18:1 (n-7), 3.3%; 18:2 (n-6), 10.5%, 18:3 (n-3), 4.8%; 18:4 (n-3), 1.2%; 20:1, 6.9%; 20:5 (n-3, EPA), 4.7%; 22:1 (n-11), 3.0% and 22:6 (n-3, DHA), 5.9%. The total content of n-3 and n-6 PUFA in the FO/RO were 17.8% and 10.9%, respectively.

The emulsifiers applied, Tween80 and Citrem LR 10 Extra (citric acid ester of mono- and diglyceride) without antioxidants were supplied by Sigma Aldrich (Steinheim, Germany) and Dupont (Danisco A/S, Grindsted, Denmark), respectively.

Alkyl caffeates were synthesized in an acid catalyzed reaction with caffeic acid and fatty alcohols with alcohol in excess as reaction medium or THF (tetrahydrofuran) as reaction medium. For further details refer to Sørensen et al. [8].

Tung oil (872 g/mol), Brij 35 (a nonionic polyoxyethylene surfactant, estimated Mw 1198 g/mol), phosphate buffer solution (PBS, pH 7.2), alumina, BHT (butylated hydroxytoluene), AAPH (2,2'-Azobis(2-methylpropionamidine) dihydrochloride) and trolox were purchased from Sigma-Aldrich

(Steinheim, Germany). Synperonic was purchased from CRODA (East Yorkshire, UK). All solvents used were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland). The external standards used for quantification of secondary oxidation products were from Sigma Aldrich (Steinheim, Germany).

Experimental design

For specific details on emulsifier, oil and antioxidants applied refer to Table 1. Experiment 1 was designed to evaluate the effect caffeic acid and different alkyl caffeates as antioxidant in Citrem stabilized emulsions in the presence of endogenous tocopherols due to the natural content of tocopherols in oil. In this experiment fish oil was used, and a storage period of 15 days selected based on previous storage experiment with fish oil.

Experiment 2 was designed to evaluate the influence of endogenous tocopherols on the efficacy of caffeic acid and caffeates in Tween stabilized emulsions. Tween was selected as emulsifier, since Citrem contains tocopherols. Emulsions were prepared with and without endogenous tocopherols. Thus, the oil without endogenous tocopherols was stripped (removal of tocopherols). Stripping of fish oil will increase oxidation rates tremendously. To slow down oxidation rate, a mixture of fish and rapeseed oil was therefore used instead in this experiment. The storage time was selected based on preliminary laboratory trials (data not shown).

Based on experiment 1 and 2 with endogenous tocopherols, the impact of emulsifier type is evaluated despite different oil type and length of storage time.

Removal of tocopherols from oils

FO/RO was stripped from tocopherols using an alumina packed glass column using hexane. For further details refer to Sørensen et al [8]. The stripped oil was bottled, flushed with nitrogen and stored at -80°C until use for production of emulsions. Furthermore, the absence of tocopherols in the oil was checked by HPLC according to the AOCS method [13]. After oil stripping (removal of tocopherols) the PV was 0.5 meq. peroxides / kg oil and tocopherols were not detected.

Production of O/W emulsions

Both in experiment 1 and 2, the emulsion compositions were 5% oil, 1% emulsifier and 94% 10 mM sodium acetate – imidazole buffer (pH 7). Antioxidants were diluted in methanol and added in concentrations of 100 µM. For the control emulsions (without antioxidant added), methanol was added in same amount as used for the methanolic antioxidant solutions added to the other emulsions. The short to medium chain phenolipids (C0 - C12) were added to the buffer (Citrem emulsions) and buffer-emulsifier mixture (Tween emulsions), whereas the long chain phenolipids (C16 – C20) were added to the oil-emulsifier mixture (Citrem emulsions) and oil (Tween emulsions) before the pre-homogenisation step.

Preparation of emulsions for storage experiment 1 were produced with pre-emulsification (2 min, Ultra-Turrax, Janke & Kunkel IKA-Labortechnik, Staufen, Germany) followed by homogenization on a two-valve table homogenizer at a pressure of 800 bar (GEA Niro Soavi Spa, Parma, Italy). For further details refer to Sørensen et al. [14]. Production of emulsions for storage experiment 2 were pre-emulsified as in experiment 1, but homogenized on a microfluidizer (9K, Microfluidics, Newton, MA, USA). Changes in the production between experiment 1 and 2 (homogenizer vs. microfluidizer) were done in order to operate with smaller emulsion volumes and thereby reduce the amount of phenolipids. Moreover, it became possible tocool the emulsion during production which

166 was an advantage due to the fact that the oil was stripped from tocopherols and would therefore be
167 highly susceptible to oxidation during homogenization.

168 After production emulsions (100 g) were stored in 100 mL blue cap bottles at 20°C ± 2°C. Samples,
169 one bottle pr. code, were taken at specific time points and divided into brown glass bottles, flushed
170 with nitrogen and stored at -40°C until analyses, except for samples used to determine droplet size,
171 these samples were measured at the sampling day without pre-freezing.

172 **Droplet size measurements**

173 Droplet size of the oil droplets in the O/W emulsion was determined by laser diffraction
174 (Mastersizer2000, Malvern Instruments Ltd., Worcestershire, UK). Few droplets of the different
175 emulsions were suspended directly in recirculating water (2800 rpm, obscuration 12-14%). Water
176 (RI_{water} = 1.330) and sunflower oil (RI_{oil} = 1.469) were used in this measurements as dispersant and
177 particle, respectively. Each sample was measured in triplicate (n=3) and results are reported as
178 surface mean diameter, $D_{3,2}$ [15].

179

180 **Lipid extractions from O/W emulsions**

181 Peroxide value (PV) and tocopherol analyses are performed on lipid extracts. Thus, the lipids were
182 extracted from the emulsions prior to these analyses according to the method described by Bligh
183 and Dyer [16] using a reduced amount of solvent [17]. For each sample code two lipid extractions
184 were performed (n=2).

185

186 **Tocopherols**

Lipid extracts were evaporated under nitrogen, re-dissolved in heptane and analyzed by HPLC (Agilent 1100 Series, Agilent Technology, Palo Alto, CA, USA) according to the AOCS Official Method Ce 8-89 [13]. A silica column (Waters (Dublin, Ireland), 150mm, 4.6mm, 3µm silica film) was used for separation of the tocopherol homologues. This analysis was performed in duplicate on each lipid extract and results reported as µg tocopherol / g emulsion.

Primary oxidation products: Peroxide value (PV)

PVs in the lipid extracts were determined by colorimetric method based on formation of an iron-thiocyanate complex. The colored complex was measured on a spectrophotometer at 500 nm (Shimadzu UV1800, Shimadzu Scientific Instruments, Columbia, MD, USA) [18]. The analysis was performed in duplicate and reported as meq peroxides / kg oil.

Secondary oxidation products: Volatiles

Volatiles were released from the O/W emulsion using dynamic headspace (45°C for 30 min, nitrogen flow of 150 mL/min). Volatiles were then collected and trapped on Tenax GR packed tubes. To avoid foam and thus water on the tubes, 4 mL of antifoam (Synperonic, conc. 8 g /L water) was added to each sample prior to the collection. Trapped volatiles were desorbed using an automatic thermal desorber (ATD-400, Perkin Elmer, Waltham, MA, USA). The transfer line of the ATD was connected to a gas chromatograph (Agilent 5890, Palo Alto, CA, USA) with a mass selective detector (HP 5972). Volatiles were separated on a DB1701 column (30m x ID 0.25mm x 1µm film thickness, J&W Scientific, Folsom, CA, USA). The initial temperature of the oven was 45°C, which was kept for 5 minutes and then gradually increased as follows: 45-55°C 1.5°C/min, 55-90°C 2.5°C/min, 90-220°C 12°C/min and kept at 220°C for 4 minutes. Calibration curves prepared from external standards was used for quantification of different volatiles. In experiment 1 (Table 1), different concentrations of external standard solutions were prepared and 1 μL was placed in Tenax tubes and analyzed. In experiment 2 (Table 1), different concentrations of external standard solutions were prepared and added to fresh emulsion without antioxidant. Volatiles were collected in the same way as for samples. The 2,4-heptadienal external standard appears as two peaks in the chromotogramme, these peaks are termed A and B. Each sample code was analyzed in triplicate (n=3) and results reported as ng volatile / g emulsion.

Partitioning

Partitioning of caffeic acid and the different alkyl caffeates in buffer/oil, emulsifier/buffer and in O/W emulsion was measured according to the method described by Schwarz et al. [19] with modifications as described elsewhere. With this method it is assumed that the partitioning of the antioxidants, equilibra reached, is not disrupted by centrifugation. For further details refer to Sørensen et al. [20]. In short, the concentration of antioxidants was measured in the separated aqueous phase of 3 different systems: buffer / oil (FO or FO/RO), buffer / emulsifier (Citrem or Tween80) and 5% O/W emulsions. Antioxidants were dissolved in methanol and added in a concentration of 100 μM. Separation of the aqueous phase was carried out 24 h after production of the different systems.

Determination of concentration of added antioxidant

Caffeic acid and alkyl caffeates in the aqueous phases were analyzed by HPLC (Agilent 1100 Series, Agilent Technology, CA, USA) with a C18 Thermo Hypersil® ODS (250x4.6 mm, 5µ) column and using a gradient elution at a flow rate of 1 mL/min. Solvent A was 3 mM phosphoric acid and solvent B was methanol. Gradient condition: 0-30 min 0-100% B, 30-40 min 100% B and 40-45 min 100-0% B. Injection volume was 20 µL. Caffeic acid and different alkyl caffeates were quantified by calibration curves of these compounds dissolved in methanol.

Conjugated autoxidizable triene (CAT) Assay

Stock solutions of the different compounds: caffeic acid, caffeates and trolox were prepared in methanol. The CAT assay was performed as described in Sørensen et al. [8] with a modification. This assay was performed with non-stripped tung oil, hence, the assay contained tocopherol and the evaluated antioxidant in a mixture. Each microplate well contained a microemulsion with the following composition: 115 µM tung oil, 17 µM Brij 35, 1 mM AAPH and antioxidant (caffeic acid, caffeates or Trolox) in various concentrations. The progress of lipid oxidation was followed by measuring the decrease in absorbance at 273 nm. Each antioxidant concentration was measured in triplicate on the plate and via independent measurements (two different microplates), n = 6. Results were expressed as CAT value (mean ± SD). This method was developed by Laguerre et al. [21]. For further details about the calculations refer to Laguerre et al. [4, 21].

Data treatment

Statistics. The results obtained were analyzed using one- and two-way ANOVA (GraphPad Prism, Version 4.01, GraphPad Software Inc). Bonferroni multiple comparison post-test was used to determine significant differences between samples or storage times. The significance level applied was 95% ($p < 0.05$). Significant difference between samples is denoted with different superscripts (i.e. a, b, c ect.).

Inhibition percentages. Since different oil and emulsifiers can influence amount of lipid oxidation, inhibition percentages were used to compare the efficacy of the antioxidants in the different emulsion systems. The antioxidants inhibition percentages were calculated according to the following equation:

$$Inhibition\ [\%] = \left(\frac{Emulsion_{Control} - Emulsion_{Antioxidant}}{Emulsion_{Control}}\right) x\ 100$$

$Emulsion_{Control}$ and $Emulsion_{Antioxidant}$ are emulsions without antioxidant and emulsion with antioxidant added, respectively. The antioxidant has a prooxidative effect if the inhibition < -5% and an antioxidative effect > 5%. The antioxidative effect can be categorized as weak (5-20%), intermediate (20-50%) or strong (> 50%) effects [22].

Multivariate data analysis, Principal Component Analysis (PCA). Inhibition percentages calculated from results obtained from PV, volatiles and tocopherol analysis were subjected to PCA using Unscrambler version X10.3 (Camo, Oslo, Norway). The PCA model was built on inhibition percentages calculated on the average of the measured data. Full cross validation was used to validate the model. A PCA allows detection of similarities and dissimilarities between the different samples in a score plot, whereas correlations between the measured variables are visualized in a loadings plot. Connecting these plots in this case shows the degree of oxidation between the different samples.

Results

Physical stability of the emulsions

The physical stability of the emulsions was evaluated by following the oil droplet size (D3,2 (surface weighted mean)) over time. With Citrem as emulsifier (Exp. 1), the droplet size was between 101 – 114 nm for the different Citrem stabilized emulsions (data not shown). The emulsion prepared with Tween as emulsifier (Exp. 2), non-stripped FO/RO and stripped FO/RO had droplet sizes between 124 – 131 nm and 126 - 131 nm, respectively (data not shown). Emulsions produced on the homogenizer (Exp. 1), resulted in slightly smaller oil droplets than emulsions produced on the microfluidizer (Exp. 2). The small differences in oil droplet sizes, however significant, for Citrem and Tween stabilized emulsions is suggested to be due to the different emulsification equipment applied, the different emulsifiers used or a combination. Changes in droplet size during storage were minor, but significant. The changes ranged from -6 nm to +2 nm in size. Since, the oil droplets mainly had a slight decrease in size during storage, the changes is suggested to be due to day to day instrumental variation. Hence, all emulsions were physically stable during the whole storage time, 42 hours, 6 and 15 days, respectively.

Partitioning of caffeic acid and caffeates

The concentration of caffeic acid (CA) and caffeates (CA C1 – CA C12) were determined in the aqueous phase of three different systems to mimic the applied emulsion systems. Hence, the two different emulsifiers and non-stripped and stripped oil was applied. The results obtained are presented in Table 2. In general, the partitioning of caffeic acid and caffeates in the aqueous phase decreased with increased degree of lipophilization until chain length C4 after which the caffeates in most cases could not be detected in the aqueous phase. In the case of the buffer/Tween system

296 octyl- (C8) and dodecyl caffeates (C12) were detected in the aqueous phase and the concentrations were not significantly different from that of butyl caffeate (C4). The concentration of caffeic acid and methyl caffeate in the aqueous phase was significantly lower when Tween was applied as emulsifier than when Citrem was applied. Moreover, the concentration of butyl caffeate tended to be lower (not significant) in the aqueous phase with Tween as emulsifier than with Citrem as also observed for caffeic acid and methyl caffeate. These partitioning results indicated interactions between antioxidants and emulsifier to a higher extent with Tween than Citrem, thus, resulting in a higher concentration of antioxidant at the interface with Tween than Citrem as emulsifier. In addition, the results indicated that there was a tendency to a lower concentration of caffeic acid and methyl caffeate in the aqueous phase of the non-stripped Tween80 emulsion, i.e. when endogenous tocopherol was present. However, the differences were not significant (Table 2).

Efficacy of caffeic acid and caffeates in emulsions

The antioxidant efficacy of caffeic acid and caffeates were evaluated in the three different emulsions shown in Table 1 and in a modified CAT assay (modification: non-stripped tung oil applied) to investigate the effect of the presence of endogenous tocopherols in this assay. The efficacy of caffeic acid and caffeates was evaluated from the measured lipid oxidation during storage. Lipid oxidation was followed by measuring PV (primary oxidation product) and volatiles (secondary oxidation products). Volatile oxidation products that increased during storage were identified and quantified. The quantified volatiles were markers of lipid oxidation products, most of them originated from oxidation of n-3 PUFAs.

Citrem stabilized emulsions with endogenous tocopherols present. The PV in this experiment increased to 20-35 meq. peroxides / kg oil depending on the antioxidant treatment during the 15 days of storage (data not shown). The concentration of volatiles after 15 days of storage dependended upon the specific volatile quantified and the antioxidant applied (1-penten-3-one 20-60 ng / g emulsion, 1-penten-3-ol 100-300 ng / g emulsion, hexanal 70-150 ng / g emulsion, 4-heptenal 10-25 ng / g emulsion, 2,4-heptadienal 4000-6000 ng / g emulsion and nonanal 30-70 ng / g emulsion).

A PCA of the PVs, α-tocopherol and volatile compounds measured during storage explained 71% of the variation in the obtained results by the first two principal components (PCs), Figure 1. The 3 other tocopherols (β-, δ-, and γ-tocopherols) were excluded from the PCA model due to no or minor changes during storage in the different emulsions. Figure 1A shows the correlation loadings i.e. graphical mapping of the measured variables. Generally, all the volatiles were located in quadrant 1 (top-right part) and 4 (bottom-right part), PVs in quadrant 2 (top-left part) closer to PC 2 and α-tocopherols in quadrant 2 and 3 (bottom-left part) a long PC 1. Thus, the first PC clearly described lipid oxidation with tocopherol in the left side and volatiles in the right side. Comparing Figure 1A with 1B, the scores, reveals the differences in efficacy between caffeic acid and caffeates. Butyl caffeate was prooxidative due to its location in the 4th quadrant i.e. high concentration of many of the volatile compounds. Octyl caffeate also acted as a prooxidant, however, not as strong as butyl caffeate. Furthermore, hexadecyl caffeate's location in first quadrant, but close to PC 2, indicates a slight prooxidative effect. Dodecyl and eicosyl caffeates seemed to have no effect due to their proximity to the control emulsion in the 2nd quadrant. The location of caffeic acid in the 3 quadrant of the scores plot away from the volatile compounds (1 and 4 quadrant) indicates that this antioxidant was the most efficient followed by methyl caffeate. However, caffeic acid and methyl caffeate were not strong antioxidants, since some of the measured volatiles were present in higher concentration in these emulsions than in the control emulsion (Table 3, Inhibition percentages, Raw data not shown).

Tween stabilized emulsions with endogenous tocopherols present. In the Tween stabilized emulsion with endogenous tocopherols present, the PV increased to 5-30 meq. peroxides / kg oil depending on the antioxidant treatment during the 6 days of storage. The concentration of volatiles after 6 days of storage dependent upon the specific volatile quantified and the antioxidant applied (1-penten-3-one 2-10 ng / g emulsion, 1-penten-3-ol 5-30 ng / g emulsion and 2,4-heptadienal 50-350 ng / g emulsion).

A PCA of the PVs, α-tocopherol and volatile compounds (1-penten-3-one, 1-penten-3-ol and 2,4-heptadienal) measured during storage explained 82% of the variation in the obtained results by the first two PCs (Figure 2). Similar to the other PCA model on Citrem stabilized emulsions, the 3 other tocopherol homologues (β-, δ-, and γ-tocopherols) were excluded from the PCA model due to no or minor changes during storage in the different emulsions. The PV and all the volatiles were located to the left in the plot of the correlation loadings (Figure 2A). Tocopherols were located opposite to the PV and volatiles. From the correlation loadings plot it is clear that the first PC describe lipid oxidation with tocopherol in the right side and volatiles in the left side, thus, increased lipid oxidation moving from the right to the left in the plot. Scores plot (Figure 2B) reveals differences in efficacy between caffeic acid and caffeates in the tween stabilized emulsions. The control emulsion was located to the left side of the PC 2 axis in the scores plot and all emulsions with antioxidant added were located to the right of the PC 2 axis except emulsion with butyl caffeate added (Figure 2B). In connection with correlation loadings plot, this indicates that all the emulsions with antioxidant added acted as antioxidant in the tween stabilized emulsions. As described above, butyl caffeate was located opposite to the PC 2 axis compared to the other emulsions with antioxidant added, this is explained by the higher amount of 2,4-heptadienal at day 6 in this emulsion (Figure 2). Butyl caffeate worked as antioxidant for all other oxidation parameters measured; however, it was the least efficient due to higher amount of the measured oxidation parameters compared to the other antioxidant applied (Table 3). Moreover, the PCA model reveals that caffeic acid without esterification was more efficient in tween stabilized emulsions followed by methyl caffeate (short chain esterification) due to their location most far away from the oxidation parameters measured. Raw data supported the PCA model.

Tween80 stabilized emulsions without endogenous tocopherols present. In the Tween stabilized emulsion with endogenous tocopherols present, the PV increased from 8 to 14 meq. peroxides / kg oil without antioxidant added during the 42 hours of storage; whereas, the PV increased from 2to 8 and 1 to 4 meq. peroxides / kg oil with caffeic acid and eicosyl caffeates, respectively The other antioxidant treatments resulted in no increase in PV during storage. The concentration of volatiles after 42 hours of storage dependent upon the specific volatile quantified and the antioxidant applied (1-penten-3-one 0-3 ng / g emulsion, 1-penten-3-ol 0-9 ng / g emulsion and 2,4-heptadienal 2-90 ng / g emulsion), which was much lower than in the Citrem stabilized emulsions.

A PCA of the PVs and volatile compounds (1-penten-3-one, 1-penten-3-ol and 2,4-heptadienal) measured during storage explained 83% of the variation in the obtained results by the first to principal components (PCs), Figure 3. All the volatiles were located in quadrant 1 (top-right part) and 4 (bottom-right part). The first PC clearly describes lipid oxidation (right side) versus no lipid oxidation (left side), whereas, PC 2 describes the development of lipid oxidation over time, with PV and volatiles in the beginning of the storage period located in the top of the plot and in the bottom of the plot after 42 days (Figure 3A). Comparing Figure 3A with 3B, the scores plot, it is observed that control emulsion and emulsion with caffeic acid is located in the same side as the oxidation parameters measured. Thus, caffeic acid is acting as a prooxidant in Tween stabilized emulsions without endogenous tocopherol present. Esterification of caffeic acid in Tween stabilized emulsion without tocopherols improved its antioxidative properties, since all caffeates evaluated were acting as antioxidants. However, it seems like caffeic acid esterified with C20 was slightly less efficient than the other esters evaluated (Table 3). Raw data supports the observation from the PCA model (data not shown).

Comparison of the influence of emulsifier type - Citrem versus Tween80 stabilized emulsions. Table 3 shows calculated inhibition percentages for caffeic acid and caffeates. It is clear that the emulsifier impacted the efficacy of the antioxidants added. In Citrem stabilized emulsions, caffeic acid and methyl caffeate were the only ones acting as antioxidants. Their antioxidative effect in this model emulsion was weak to intermediate and they even promoted the formation of certain volatiles. In contrast, caffeic acid and all the evaluated caffeates in Tween stabilized emulsions acted as antioxidants. Caffeic acid was the strongest antioxidant followed by methyl caffeate.

Comparison of the influence of the presence of endogenous tocopherols. Calculated inhibition percentages for selected oxidation variables measured in Tween stabilized emulsions with and without endogenous tocopherols are presented in Table 3. A clear difference in the efficacy of antioxidants was that caffeic acid acted as a strong antioxidant when tocopherols were present, whereas, it acted as intermediate to strong prooxidant without tocopherols in this model emulsion. In addition, the caffeates acted as stronger antioxidants without tocopherols compared to the same model emulsion with tocopherols present (Table 3). Caffeates with chain lengths between C1 and C18 were all strong antioxidants, whereas the antioxidant efficacy was decreased when the chain length was increased to C20.

Antioxidant efficacy in a modified CAT assay. The CAT assay is an assay developed to measure the efficacy of antioxidants in a micro emulsion system without endogenous tocopherols present. The assay was slightly modified to investigate the efficacy of caffeic acid and caffeates in the presence of endogenous tocopherols. The results are shown in Figure 4 together with results obtained earlier with an unmodified CAT assay [8]. When tocopherol was present the antioxidative

414 efficacies of caffeic acid, methyl-, butyl-, octyl- and dodecyl caffeates were not significantly different. This finding is different from earlier results obtained without the presence of tocopherols, where octyl- and dodecyl caffeates exerted a significantly higher efficacy than caffeic acid and the other caffeates evaluated. Furthermore, the efficacy of octyl- and dodecyl caffeates without tocopherols present was also significantly higher than when tocopherols were present.

Discussion

The results showed - as hypothesized - that partitioning of the antioxidants was affected by the type of emulsifier and the presence of endogenous tocopherols. Earlier measurements of the radical scavenging effect (DPPH assay) of caffeic acid and caffeates showed no differences between the caffeic acid and the different saturated unbranched alkyl esters [8]. In spite of that the antioxidative effect of caffeic acid and caffeates was different in the different emulsion systems. However, the activity can differ due to the emulsion composition.

Impact of emulsifier on the efficacy of caffeic acid and caffeates. In the present study, caffeic acid and caffeates were more efficient antioxidants in Tween stabilized emulsions than in Citrem stabilized emulsions. This partly supports earlier findings with the same emulsifiers and caffeic acid in 10% O/W emulsions, where caffeic acid in Citrem stabilized emulsions promoted the formation of volatiles and no effect on lipid oxidation or slightly antioxidative effect of caffeic acid was observed in Tween stabilized emulsions [23]. Besides different oil concentration in the previous and current studies, antioxidant concentration (5.5 fold lower in this study) and oil / emulsifier ratio were also different, which can have an influence on the differences observed in these studies. Independently of emulsifier type, caffeic acid was performing better than the caffeates. Contrary, the effect of the caffeates was affected by the emulsifier applied. To our knowledge only few studies related to the antioxidative effect of caffeic acid and caffeates in emulsions have been published [9, 24, 25]. These studies did not compare the effect of the emulsifier type applied. However, one of them compared the effect of caffeates in mayonnaise and milk, where not only the emulsifier type is different but the entire emulsion system. The efficiency of the caffeates was affected by the type of emulsion system [9]. Results obtained in the present study also demonstrated that changing the emulsifier affected the antioxidative effect and rank order of caffeates. Experiments performed with gallic acid and ethyl gallate have also shown that changing emulsifier affects the partitioning of the antioxidants and the resulting antioxidant activity measured in emulsion systems [26]. The different emulsifier evaluated was SDS, CTAB, Brij58 and PHLC, and their partitioning study revealed increased solubility effect of the emulsifiers in the following order: PHLC < SDS < Brij58 < CTAB. The antioxidant activity of gallic acid and ethyl gallate based on the formation of hydroperoxides and hexanal increased in the following order: CTAB (no activity measured) < Brij58 < PHLC < SDS. Gallic acid only showed antioxidant activity with PHLC stabilized emulsions. This was a reverse order compared to the partitioning measured. Hence, it was suggested that the increased partitioning into the emulsifier layer and lipid counteract the hydrogen-donating ability, and lower the activity of the antioxidants [26]. Moreover, Pekkarinen et al. [27] evaluated antioxidative effect and partitioning of phenolics in different systems. Interaction between caffeic acid and Tween 20 differed from other phenolics such as vanillic acid, ferulic acid and sinapic acid evaluated, since Tween 20 exhibited higher solubilisation capacity for caffeic acid than for other phenolic acids. Additionally, Pekkarinen et al. [27] concluded that these antioxidant-emulsifier interactions have a strong influence on the partitioning of antioxidants. The partitioning result obtained in this study confirmed that Citrem and Tween as emulsifiers results in differences in the partitioning of caffeic acid and caffeates. Less caffeic acid and caffeates were present in the aqueous phase when Tween was applied. This clearly demonstrated stronger antioxidant-emulsifier interaction with Tween compared to Citrem. Moreover, Citrem is an anionic emulsifier, thus, the interface is negatively charged and will repel negatively charged antioxidants i.e. caffeic acid, which could explain why caffeic acid interacted less with Citrem than with Tween. An explanation for the stronger interactions with Tween may be the molecular structure of the emulsifiers, since Tween is a larger and more bulky molecule than Citrem; however, this has to be further evaluated. Furthermore, Schwarz et al. [19, 28, 29] evaluated partitioning of different antioxidants in dispersed lipid systems with different emulsifiers. Significant differences were observed in partitioning of the antioxidants between phases, both as a function of pH and emulsifier type and concentration. It was concluded from the results that determination of antioxidant partitioning may be an important tool to select antioxidants structurally designed to localize at the surfaces [19], however, the partitioning of the antioxidants cannot alone explain the measured antioxidant activity in emulsions [29]. Results from another study by Schwarz et al. [30] evaluating antioxidant activity of antioxidants with different lipophilicity in bulk oil, O/W and W/O emulsions with different emulsifiers led to the assumption that differences in antioxidant activity for the same emulsion type might be additionally influenced by interaction with the emulsifier dominating the interfaces in the emulsion system [30]. The obtained results for the two emulsifiers together with the partitioning study may also here lead to the assumption that emulsifier-antioxidant interactions (e.g. hydrogen bonding) affected the antioxidant activity of the caffeates. However, the type of interactions and the impact of antioxidant-emulsifier interactions on the radical scavenging activity have to be studied in more details to make further conclusions.

Impact of endogenous tocopherols on the efficacy of caffeic acid and caffeates. The presence of endogenous tocopherol not only changed the antioxidant activity of caffeic acid and caffeates in both the storage experiment and in the CAT assay, but also their partitioning in the emulsion system. A tendency to less caffeic acid and caffeates (C1, C4 and C12) present in the aqueous phase with endogenous tocopherol in the emulsion system was observed. This may indicate some interactions between tocopherol and caffeic acid / caffeates both for the antioxidative effect and localization in the emulsion system. The use of a combination of antioxidants to produce synergistic interaction has been reported earlier e.g. tocopherol regeneration by ascorbic acid, polyphenols and flavonoids [31-34]. Panya et al. [34] carried out the only study investigating interactions between tocopherol and a phenol (rosmarinic acid) and its alkyl esters (rosmarinates, C4, C12 and C20) in Tween20 stabilized emulsions. Rosmarinic acid exhibited strongest synergistic interaction with

491 tocopherol, and C4 and C12 esters exhibited small synergistic interaction. An antagonistic
492 interaction was observed with C20 ester and tocopherol. Thus, the more hydrophilic rosmarinic acid
493 exhibited more interactions with the tocopheryl radical than the esters. In the present study, the
494 emulsion with the more hydrophilic caffeic acid exhibited better oxidative stability than the
495 emulsions with the esters (more hydrophobic antioxidants) when tocopherol was present as also
496 observed with rosmarinic acid and rosmarinates. Actually, caffeic acid turned from being
497 prooxidative without tocopherol present to being the most efficient antioxidant with endogenous
498 tocopherol present in Tween stabilized emulsion. In emulsions, the majority of the emulsifier is
499 accumulated at the oil-water interface. However, a part of the emulsifier is not associated with the
500 oil-water interface if the emulsifier concentration is above the CMC (critical micellar concentration,
501 CMC Tween80 13-15 mg/L, Sigma) and will form micelles in the aqueous phase. In this study, the
502 concentration of Tween was much higher than CMC (10 g/L). The decreased antioxidant efficiency
503 of the different caffeates compared with caffeic acid is suggested to be due to the solubilisation of
504 caffeates in Tween micelles, thus, localized away from the interface unable to inhibit lipid
505 oxidation. Although, micelles are not isolated structures, they compromise structures that are in
506 dynamic equilibrium with other structures in the emulsion system. This means that components can
507 be exchanged between the different structures i.e. between micelles and emulsion droplets [35, 36].
508 Thus, more studies are needed to further elucidate the differences observed in partitioning and
509 antioxidant effect in the presence of endogenous tocopherols.

510 Additionally, it is assumed that caffeic acid is located in close proximity to the interface where it
511 regenerates tocopherol at the interface in spite of the repelling effect of two negatively charged
512 compounds (Citrem and caffeic acid). The proposed partial location of tocopherol at the interface is
513 supported by Jacobsen et al. [37] who reported that ca. 6 % of the alpha-tocopherol present in
514 mayonnaise was located at the interface whereas the remaining tocopherol was located in the oil

phase. Therefore, lipophilization is not needed to improve the oxidative stability of these emulsions, due to tocopherols location at the interface. ***Antioxidant hypotheses and efficacies of caffeic acid and caffeates.*** The polar paradox was not confirmed in this study since caffeic acid was more efficient as antioxidant than caffeates in Citrem stabilized emulsions when endogenous tocopherol was present. In Tween stabilized emulsions with endogenous tocopherol caffeic acid followed by methyl caffeate were the most efficient antioxidants, whereas, caffeic acid acted as a prooxidant when tocopherol was not present. No cut-off effect was observed for the lipophilized caffeic acid, since the most efficient antioxidant in both Citrem and Tween stabilized emulsions when tocopherol was present was C0 (caffeic acid). A similar finding was observed for the CAT assay with endogenous tocopherol present. Without endogenous tocopherol present, the caffeates were most efficient antioxidants in Tween stabilized emulsions, a cut-off effect was found at C16. However, the efficiency of C20 was still an intermediate to strong antioxidant. The CAT assay showed a cut-off effect at around C8 and C12 with no endogenous tocopherol present. The partitioning experiment clearly showed an effect of the chain length, with less antioxidant present in the aqueous phase with increasing antioxidant lipophilicity. Pekkarinen et al. [27] observed that the proportion of antioxidant solubilized in the lipid phase and particularly in the interface did not necessarily reflect the efficiency of the antioxidant. It was assumed from their evaluation of antioxidant activity and partitioning that specific interactions of the antioxidant with other compounds e.g. emulsifiers, and intermolecular hydrogen bonds may play an important role in reducing antioxidant activity. Furthermore, antioxidant-emulsifier interaction has a strong influence on partitioning of the antioxidant. It is suggested that the caffeates interacts with the emulsifier in form of micelles when endogenous tocopherols are present, resulting in reduced or no antioxidant activity. When endogenous tocopherols are not present the caffeates is more likely to be solubilized at the interface instead of the tocopherols. However, this has to be evaluated more in depth to conclude further on these differences in partitioning and antioxidant activity with the impact of endogenous tocopherols.

Conclusions. Partitioning and antioxidant activity of caffeic acid and caffeates were influenced both by the emulsifier type and the presence of endogenous tocopherols. Thus, this study clearly demonstrated different emulsifier-antioxidant and antioxidant-antioxidant interactions that affected the efficacy of the evaluated caffeic acid and caffeates as antioxidant in emulsions. The hypotheses about antioxidant in emulsions are based on simple emulsions systems without the presence of tocopherols. However, the impact of the presence of tocopherols on the efficacy of other antioxidants is important since most food systems contain tocopherol.

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Figure legends

Figure 1 PCA build on results obtained from PV, volatiles (1-penten-3-one, 1-penten-3-ol, 4-heptenal, 2,4-heptadienal, hexanal and nonanal) and α-tocopherols measured on Citrem stabilized emulsions during storage (15 days) using full cross validation. A) Correlation loadings and B) Scores plot. Abbreviations for sample codes refer to Table 1.

Figure 2 PCA build on results obtained from PV, volatiles (1-penten-3-one, 1-penten-3-ol and 2,4-heptadienal) and α-tocopherols measured on Tween80 stabilized emulsions during storage (6 days) using full cross validation. A) Correlation loadings and B) Scores plot. Abbreviations for sample codes refer to Table 1.

Figure 3 PCA build on results obtained from PV and volatiles (1-penten-3-one, 1-penten-3-ol and 2,4-heptadienal) measured on Tween80 stabilized emulsions during storage (42 hours) using full cross validation. A) Correlation loadings and B) Scores plot. Abbreviations for sample codes refer to Table 1.

Figure 4 CAT Value of caffeic acid and caffeates (C1-C16) measured in the concentration range of 0.5 – 2 µM. ● CAT Values determined without endogenous tocopherols (normal condition for the CAT assay, published in Sørensen et al. [8]) and ○ CAT Values determined with endogenous tocopherols (modified CAT assay).

Table 1 Experimental design of experiment 1 and 2.

Experiment	Sample code	Emulsifier	Oil	Antioxidant
EXP 1	C_Con	Citrem	FO	*No antioxidant*
	C_CA C0	Citrem	FO	*Caffeic acid*
	C_CA C1	Citrem	FO	*Methyl caffeate*
	C_ CA C4	Citrem	FO	*Butyl caffeate*
	C_CA C8	Citrem	FO	*Octyl caffeate*
	C_CA C12	Citrem	FO	*Dodecyl caffeate*
	C_CA C16	Citrem	FO	*Hexadecyl caffeate*
	C_ CA C20	Citrem	FO	*Eicosyl caffeate*
EXP 2	T_Con	Tween80	FO/RO	*No antioxidant*
	T_CA C0	Tween80	FO/RO	*Caffeic acid*
	T_ CA C1	Tween80	FO/RO	*Methyl caffeate*
	T_ CA C4	Tween80	FO/RO	*Butyl caffeate*
	T_ CA C8	Tween80	FO/RO	*Octyl caffeate*
	T_CA C12	Tween80	FO/RO	*Dodecyl caffeate*
	T_CA C16	Tween80	FO/RO	*Hexadecyl caffeate*
	TS_Con	Tween80	S FO/RO	*No antioxidant*
	TS_CA C0	Tween80	S FO/RO	*Caffeic acid*
	TS_CA C1	Tween80	S FO/RO	*Methyl caffeate*
	TS_ CA C4	Tween80	S FO/RO	*Butyl caffeate*
	TS_CA C8	Tween80	S FO/RO	*Octyl caffeate*
	TS_CA C12	Tween80	S FO/RO	*Dodecyl caffeate*
	TS_CA C16	Tween80	S FO/RO	*Hexadecyl caffeate*
	TS_CA C20	Tween80	S FO/RO	*Eicosyl caffeate*

Abbreviations: FO Fish oil, FO/RO Fish oil and rapeseed oil (1:1, w/w) and S FO/RO Stripped fish oil and rapeseed oil (1:1, w/w)

Table 2 Concentration [µM] of caffeic acid and caffeates (Methyl, Butyl, Octyl and Dodecyl) measured in the aqueous phase of different systems: 95% Buffer / 5% Oil, 99% Buffer / 1% Emulsifier and Emulsion (5% Oil, 1% Emulsifier and 94% Buffer). Citrem and Tween80 were applied as emulsifier. Both non-stripped and stripped FO/RO was evaluated with Tween80 as emulsifier.

Antioxidant	Citrem and non-stripped oil			Tween80 and non-stripped oil			Tween80 and stripped oil	
	Buffer / Oil	**Buffer / Emulsifier**	**Emulsion**	**Buffer / Oil**	**Buffer / Emulsifier**	**Emulsion**	**Buffer / Oil**	**Emulsion**
CA C0	101 ± 3.1^{a,b,x}	91.5 ± 3.6^{b,x}	91.5 ± 7.1^{b,x}	93.7 ± 11.4^{b,x}	75.4 ± 7.0^{c,x}	74.0 ± 4.3^{c,x}	111 ± 2.6^{a,x}	78.4 ± 10.1^{c,x}
CA C1	86.2 ± 0.9^{a,y}	53.3 ± 3.0^{b,y}	43.8 ± 8.2^{b,y}	82.8 ± 1.9^{a,x}	15.4 ± 3.3^{c,y}	10.1 ± 2.5^{c,y}	91.1 ± 2.6^{a,y}	15.3 ± 3.1^{c,y}
CA C4	11.4 ± 0.4^{a,b,z}	4.09 ± 0.6^{a,b,c,z}	3.39 ± 0.5^{b,c,z}	11.3 ± 0.7^{a,b,y}	2.10 ± 0.3^{c,z}	< detection	13.0 ± 1.1^{a,z}	< detection
CA C8	< detection	< detection	< detection	< detection	2.60 ± 3.6^{z}	< detection	< detection	< detection
CA C12	< detection	< detection	< detection	2.00 ± 0.6^{b,z}	1.10 ± 0.4^{z}	< detection	9.70 ± 4.2^{a,z}	1.10 ± 0.2^{b,y}

Different letters in superscript indicate significant differences. Significant differences within a row i.e. same antioxidant but different systems are denoted with a,b and c, whereas significant differences within a column i.e. same system but different antioxidant are denoted with x, y and z.

Table 3 Calculated inhibition percentages for selected lipid oxidation parameters (PV, 1-penten-3-ol, 1-penten-3-one and 2,4-heptadienalA) measured at selected time points in the different emulsions. Citrem stabilized emulsions are reported at day 6 and 15, Tween stabilized emulsions with endogenous tocopherols at day 6 and Tween stabilized emulsions without endogenous tocopherols at 42 hours.

AO	Citrem (Day 6)				Tween with tocopherols				Tween without tocoherols			
	PV	1Pen3ol	1Pen3one	2,4HepA	PV	1Pen3ol	1Pen3one	2,4HepA	PV	1Pen3ol	1Pen3one	2,4HepA
CA	50	39	3	44	79	83	86	86	34	-63	-55	-48
C1	32	1	-210	-27	60	60	69	11	89	97	85	89
C4	21	-119	-502	-143	47	61	33	-10	89	99	83	93
C8	-28	-174	-603	-87	49	71	52	-3	88	100	94	94
C12	-53	-130	-515	-13	43	62	55	2	91	102	97	95
C16	-26	-104	-487	-39	44	58	39	4	87	101	96	98
C20	-31	-43	-340	8					66	59	44	45
AO	Citrem (Day 15)											
	PV	1Pen3ol	1Pen3one	2,4HepA								
CA	13	-166	52	13								
C1	31	-123	65	18								
C4	28	-61	-65	-41								
C8	54	-47	-51	-17								
C12	5	-10	-28	5								
C16	-7	3	-21	5								
C20	-5	12	-15	7								

Abbreviation: AO Antioxidant; PV Peroxide Value; 1Pen3ol 1-Penten-3-ol; 1Pen3one 1-Penten-3-one; 2,4HepA 2,4-HeptadienalA. The 2,4-heptadienal external standard appears as two peaks in the chromotogramme, these peaks are termed A and B (here only 2,4HepA presented).

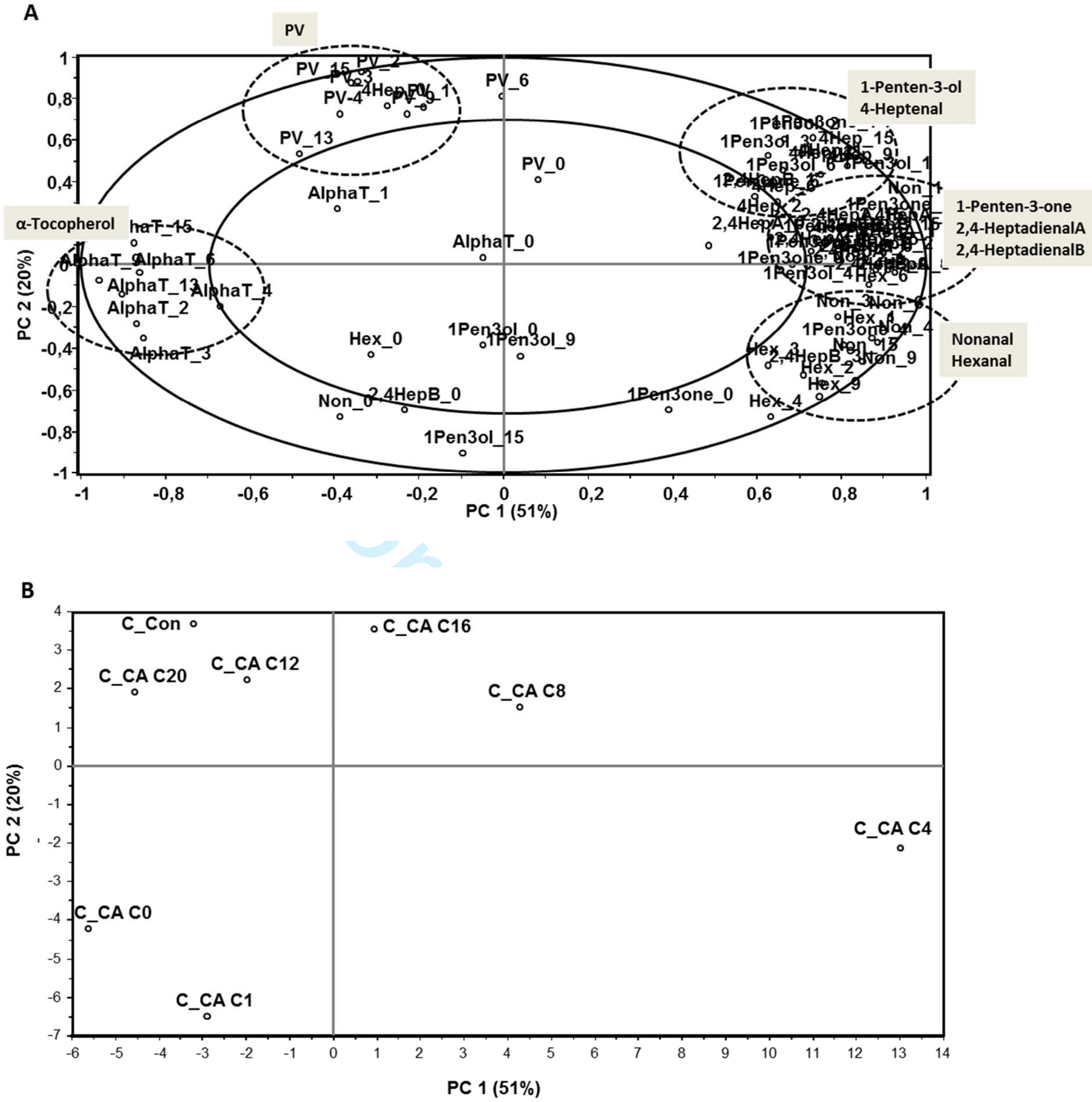
A
PV
1-Penten-3-ol
4-Heptenal
α-Tocopherol
1-Penten-3-one
2,4-HeptadienalA
2,4-HeptadienalB
Nonanal
Hexanal
PV_13
PV_6
PV_0
AlphaT_1
AlphaT_0
AlphaT_4
AlphaT_2
AlphaT_3
Hex_0
1Pen3ol_0
1Pen3ol_9
Non_0
2,4HepB_0
1Pen3one_0
1Pen3ol_15
PC 2 (20%)
PC 1 (51%)
B
C_Con
C_CA C20
C_CA C12
C_CA C16
C_CA C8
C_CA C4
C_CA C0
C_CA C1
PC 2 (20%)
PC 1 (51%)

Figure 1

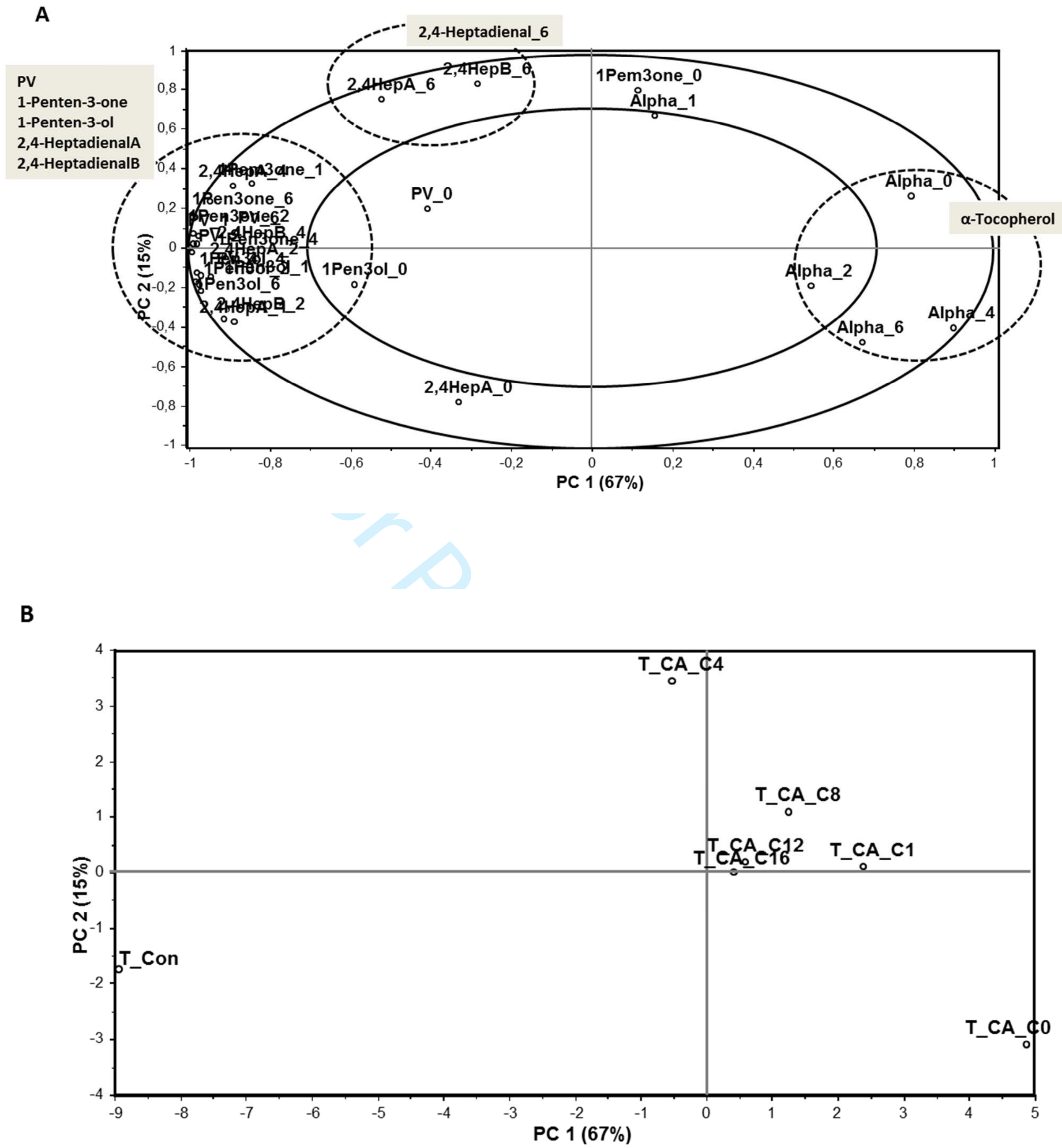
A
2,4-Heptadienal_6
PV
1-Penten-3-one
1-Penten-3-ol
2,4-HeptadienalA
2,4-HeptadienalB
α-Tocopherol
2,4HepA_6
2,4HepB_6
1Pem3one_0
Alpha_1
PV_0
1Pen3ol_0
Alpha_0
Alpha_2
Alpha_6
Alpha_4
2,4HepA_0
1Pen3one_6
1Pen3one_1
Pen3ol_6
PC 2 (15%)
PC 1 (67%)
B
T_CA_C4
T_CA_C8
T_CA_C12
T_CA_C16
T_CA_C1
T_Con
T_CA_C0
PC 2 (15%)
PC 1 (67%)

Figure 2

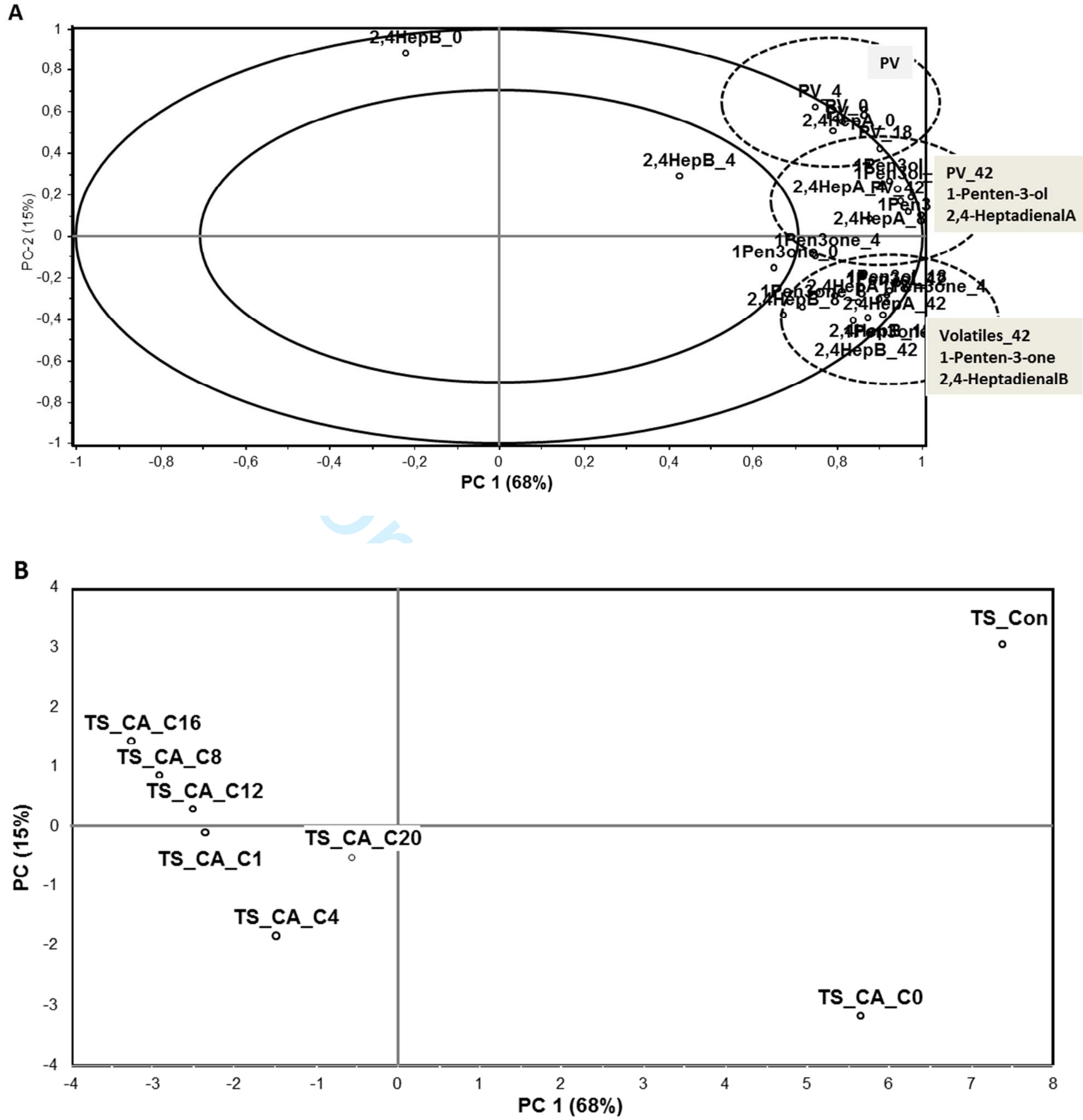
A
2,4HepB_0
PV
PV_4
PV_18
2,4HepB_4
PV_42
1-Penten-3-ol
2,4-HeptadienalA
2,4HepA_8
1Pen3one_4
1Pen3one_0
2,4HepA_42
2,4HepB_42
Volatiles_42
1-Penten-3-one
2,4-HeptadienalB
PC-2 (15%)
PC 1 (68%)
B
TS_Con
TS_CA_C16
TS_CA_C8
TS_CA_C12
TS_CA_C20
TS_CA_C1
TS_CA_C4
TS_CA_C0
PC (15%)
PC 1 (68%)

Figure 3

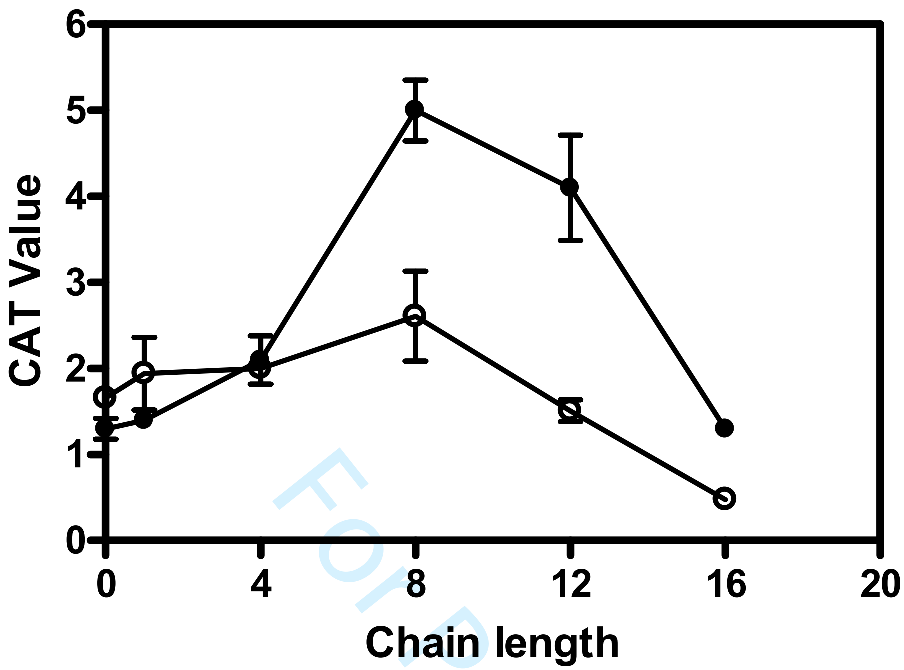

Figure 4

Alkyl caffeates as antioxidants in ~~o/w~~O/W emulsions: Impact of emulsifier type and endogenous tocopherols

Ann-Dorit Moltke Sørensen[1]*, Pierre Villeneuve[2], Charlotte Jacobsen[1]

[1] Division of Food Technology, National Food Institute (DTU Food), Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

[2] CIRAD, UMR IATE, Montpellier, F-34398, France

[*] Corresponding author: adms@food.dtu.dk, Phone: +45 4525 2591

Running title: ~~Oxidation is affected by emulsifier and antioxidant~~ Effect of emulsifier and antioxidants on lipid oxidation

Keywords: Caffeic acid; Lipid oxidation; Tween; Citrem; Phenolipids; Fish oil; Antioxidant interactions

Abbreviations: CA Caffeic acid; C1 Methyl caffeate; C4 Butyl caffeate; C8 Octyl caffeate; C12 Dodecyl caffeate; C16 Hexadecyl caffeate; C20 Eicosyl caffeate; CAT Conjugated autoxidizable triene; CCL Critical chain length; FO Fish oil; FO/RO Fish oil and rapeseed oil mixture (1:1); PC Principal component; PCA Principal component analysis; PV Peroxide value; THF Tetrahydrofuran

Abstract

Antioxidant addition can be one strategy to limit lipid oxidation in emulsions. Research has proven that an important factor regarding the efficacy of antioxidants is their localization in the emulsion; however, other factors such as interactions with other components can also have an impact. Thus, the aim was to evaluate the impact of emulsifiers (Citrem and Tween80) and presence of endogenous tocopherols on the efficacies of caffeic acid and caffeates (C1-C20) as antioxidants in emulsions. Lipid oxidation was evaluated during storage and partitioning of caffeic acid and caffeates was estimated by measuring their concentrations in the aqueous phase.

Partitioning of caffeic acid and caffeates was influenced by emulsifier type and presence of endogenous tocopherols. Caffeic acid was the most efficient antioxidant in Citrem and Tween stabilized emulsions in the presence of endogenous tocopherol. In contrast, for Tween stabilized emulsions, caffeic acid acted as a prooxidant and the evaluated caffeates acted as strong antioxidants in the absence of endogenous tocopherol. Thus, when endogenous tocopherol was present lipophilization of caffeic acid did not increase its efficacy as an antioxidant. It is suggested that the differences observed in antioxidant efficiency with different emulsifiers and with and without endogenous tocopherols is due to emulsifier-antioxidant interactions and antioxidant-antioxidant interactions in the emulsions.

Practical application: Food emulsions contain endogenous tocopherols, thus, the impact of endogenous tocopherols on the efficacy of applied antioxidants is of interest to the industry. So far the hypotheses about antioxidant in emulsions are based on simple emulsions systems without the presence of tocopherols. The finding in this study revealed that both emulsifier type and the presence of endogenous tocopherol had an impact on the efficacy of caffeic acid and caffeates due to emulsifier-antioxidant and antioxidant-antioxidant interactions. This highlights the importance of

evaluating the antioxidant in each emulsion system before selecting antioxidants for optimal protection against lipid oxidation.

Introduction

An o/w emulsion consists of three different phases. A dispersed phase (oil) is present as droplets in a continuous phase (water) and separated by an interfacial region. Type of emulsions can range from very simple, when prepared from only a few ingredients, to more complex, when prepared with many different ingredients e.g. food emulsions. Lipid oxidation can occur rapidly in emulsions due to their large interfacial area. The interface region facilitates interactions between the lipids and water-soluble prooxidants [1].

Different strategies can be applied to limit lipid oxidation and thereby improve shelf life of emulsions [2]. One of them is addition of antioxidants; however, selection of the right antioxidant or mixture of antioxidants is difficult, since their efficacies are affected by the composition of the emulsions e.g. their localization and interaction with other components. An enormous amount of studies have been reported in the literature on antioxidants and their efficacies in model emulsions and more complex food emulsions. So far, two hypotheses about antioxidant efficacies in emulsions, namely the polar paradox hypothesis [3] and the cut-off effect [4] have been reported. In brief, the polar paradox hypothesizes that apolar antioxidants are more efficient in O/W emulsions than polar antioxidants [3] due to differences in the antioxidants' affinity towards the different phases [5]. The cut-off effect can be seen as an extension of the polar paradox hypothesis, in which an optimal degree of lipophilisation for optimal antioxidant activity is observed [4], which is called critical chain length (CCL). The cut-off effect was observed from results obtained with chlorogenic acid and rosmarinic acid and their unbranched saturated alkyl esters (chlorogenates and rosmarinates). The efficacy of antioxidant homologues was related to the partitioning of these antioxidants in an emulsion system [4, 6]. Based on these observations, it was assumed that when the lipophilized antioxidants had the CCL they were present in the highest concentration at the oil-aqueous interface, where lipid oxidation is initiated. In addition, antioxidant homologues with chain length below and above CCL were driven away from the oil-aqueous interface [7]. These antioxidant hypotheses are based on extensive research in simplified emulsions prepared with stripped oils, whereas food emulsions contain e.g. endogenous tocopherols. Only few compounds with different degree of lipophilization have been evaluated in several different emulsion systems. Caffeic acid and unbranched saturated caffeates have been evaluated both in a model emulsion (CAT assay) system [8], milk and mayonnaise [9] and CCL seemed to be influenced by the system. In addition, rosmarinic acid has also been evaluated in different systems; model emulsions and low moisture food (crackers). In model emulsions, a parabolic relationship between antioxidant efficacy and hydrophobicity was observed, with the intermediate polarity (8 carbon – 18 carbon chain length) giving optimum activity [6, 10, 11]. In crackers, a linear relationship between antioxidant activity and hydrophobicity was observed [12].

Due to formerly obtained results with caffeic acid and caffeates in model emulsion, milk and mayonnaise [8, 9], it is hypothesized that emulsifier type and the presence of endogenous tocopherols can affect the partitioning of caffeic acid and caffeates and thereby change their efficacy in the emulsion systems. Hence, the aim of this study was to evaluate the impact of both emulsifier and presence of endogenous tocopherols on the efficacies of caffeic acid and caffeates (C1-C20). Two experiments were carried out as shown in Table 1. In the first experiment, the aim was to evaluate the effect of lipophilization of caffeic acid on its antioxidant efficacy in an ~~o/w~~O/W emulsion prepared with unstripped oil and Citrem as emulsifier. The aim of the second experiment was to investigate whether the presence of endogenous tocopherol affected the optimal chain length of lipophilized caffeic acid when Tween was used as an emulsifier. Moreover, comparison of results of the two experiments enabled an evaluation of whether the emulsifier type, Citrem vs Tween affected the CCL in emulsions prepared with unstripped oil.

In both experiments, the partitioning of the antioxidant was estimated by measuring its concentration in the aqueous phase of the ~~o/w~~O/W emulsion, a buffer/oil system and a buffer/emulsifier system to evaluate if emulsifier type and the presence of endogenous tocopherols, affected the partitioning of caffeic acid and caffeates in the emulsion system.

Material and Methods

The oil used in this study was fish oil (FO) and a mixture between fish and rapeseed oil (FO/RO, 1:1). The two types of oil were supplied by Maritex A/S (TINE BA, Sortland, Norway). The quality of the FO was as follows: 0.3 meq. peroxides / kg oil, 250 mg α-tocopherol / kg, 98 mg γ-tocopherol / kg and 48 mg δ-tocopherol / kg. Fatty acid composition of the fish oil 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-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, 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 the FO were 24.0% and 1.8 %, respectively. The quality of the FO/RO was as follows: 0.3 meq. peroxides / kg oil, 230 mg α-tocopherol / kg, 31 mg β-tocopherol / kg, 151 mg γ-tocopherol / kg and 6 mg δ-tocopherol / kg. The fatty acid composition of the FO/RO was as follows: 14:0, 1.7%; 16:0, 7.1%; 16:1 (n-7), 4.6%; 18:0, 1.9%; 18:1 (n-9), 38.3%; 18:1 (n-7), 3.3%; 18:2 (n-6), 10.5%, 18:3 (n-3), 4.8%; 18:4 (n-3), 1.2%; 20:1, 6.9%; 20:5 (n-3, EPA), 4.7%; 22:1 (n-11), 3.0% and 22:6 (n-3, DHA), 5.9%. The total content of n-3 and n-6 PUFA in the FO/RO were 17.8% and 10.9%, respectively.

The emulsifiers applied, Tween80 and Citrem LR 10 Extra (citric acid ester of mono- and diglyceride) without antioxidants were supplied by Sigma Aldrich (Steinheim, Germany) and Dupont (Danisco A/S, Grindsted, Denmark), respectively.

Alkyl caffeates were synthesized in an acid catalyzed reaction with caffeic acid and fatty alcohols with alcohol in excess as reaction medium or THF (tetrahydrofuran) as reaction medium. For further details refer to Sørensen et al. [8].

Tung oil (872 g/mol), Brij 35 (a nonionic polyoxyethylene surfactant, estimated Mw 1198 g/mol), ~~AAPH (2,2'-azobis-2-methyl-propanimidamide, dihydrochloride),~~ phosphate buffer solution (PBS, pH 7.2), alumina, BHT (butylated hydroxytoluene), AAPH (2,2'-Azobis(2-methylpropionamidine) dihydrochloride) and trolox were purchased from Sigma-Aldrich (Steinheim, Germany). Synperonic was purchased from CRODA (East Yorkshire, UK). All solvents used were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland). The external standards used for quantification of secondary oxidation products were from Sigma Aldrich (Steinheim, Germany).

Experimental design

For specific details on emulsifier, oil and antioxidants applied refer to Table 1. Experiment 1 was designed to evaluate the effect caffeic acid and different alkyl caffeates as antioxidant in Citrem stabilized emulsions in the presence of endogenous tocopherols due to the natural content of tocopherols in oil. In this experiment fish oil was used, and a storage period of 15 days selected based on previous storage experiment with fish oil.

Experiment 2 was designed to evaluate the influence of endogenous tocopherols on the efficacy of caffeic acid and caffeates in Tween stabilized emulsions. Tween was selected as emulsifier, since Citrem contains tocopherols. Emulsions were prepared with and without endogenous tocopherols. Thus, the oil without endogenous tocopherols was stripped (removal of tocopherols). Stripping of fish oil will increase oxidation rates tremendously. To slow down oxidation rate, a mixture of fish and rapeseed oil was therefore used instead in this experiment. The storage time was selected based on preliminary laboratory trials (data not shown).

Based on experiment 1 and 2 with endogenous tocopherols, the impact of emulsifier type is evaluated despite different oil type and length of storage time.

Removal of tocopherols from oils

FO/RO was stripped from tocopherols using an alumina packed glass column using hexane. For further details refer to Sørensen et al [8]. The stripped oil was bottled, flushed with nitrogen and stored at -80°C until use for production of emulsions. Furthermore, the absence of tocopherols in the oil was checked by HPLC according to the AOCS method [13]. After oil stripping (removal of tocopherols) the PV was 0.5 meq. peroxides / kg oil and tocopherols were not detected.

Production of ~~o/w~~O/W emulsions

Both in experiment 1 and 2, the emulsion compositions were 5% oil, 1% emulsifier and 94% 10 mM sodium acetate – imidazole buffer (pH 7). Antioxidants were diluted in methanol and added in concentrations of 100 µM. For the control emulsions (without antioxidant added), methanol was added in same amount as used for the methanolic antioxidant solutions added to the other emulsions. The short to medium chain phenolipids (C0 - C12) were added to the buffer (Citrem emulsions) and buffer-emulsifier mixture (Tween emulsions), whereas the long chain phenolipids (C16 – C20) were added to the oil-emulsifier mixture (Citrem emulsions) and oil (Tween emulsions) before the pre-homogenisation step.

Preparation of emulsions for storage experiment 1 were produced with pre-emulsification (2 min, Ultra-Turrax, Janke & Kunkel IKA-Labortechnik, Staufen, Germany) followed by homogenization on a two-valve table homogenizer at a pressure of 800 bar (GEA Niro Soavi Spa, Parma, Italy). For further details refer to Sørensen et al. [14]. Production of emulsions for storage experiment 2 were pre-emulsified as in experiment 1, but homogenized on a microfluidizer (9K, Microfluidics, Newton, MA, USA). Changes in the production between experiment 1 and 2 (homogenizer vs. microfluidizer) w~~as~~ere done in order to operate with smaller emulsion volumes and~~,~~ thereby reduce the amount of phenolipids. Moreover, it became possible to~~, and the ability to~~ cool the emulsion during production which was an advantage due to the fact that ~~when~~ the oil was stripped from tocopherols and would therefore be highly susceptible to oxidation during homogenization.

After production emulsions (100 g) were stored in 100 mL blue cap bottles at 20°C ± 2°C. Samples, one bottle pr. code, were taken at specific time points and divided into brown glass bottles, flushed with nitrogen and stored at -40°C until analyses, except for samples used to determine droplet size, these samples were measured at the sampling day without pre-freezing.

Droplet size measurements

Droplet size of the oil droplets in the ~~o/w~~O/W emulsion was determined by laser diffraction (Mastersizer2000, Malvern Instruments Ltd., Worcestershire, UK). Few droplets of the different emulsions were suspended directly in recirculating water (2800 rpm, obscuration 12-14%). Water (RI_{water} = 1.330) and sunflower oil (RI_{oil} = 1.469) were used in this measurements as dispersant and particle, respectively. Each sample was measured in triplicate (n=3) and results are reported as surface mean diameter, $D_{3,2}$ [15].

Lipid extractions from ~~o/w~~O/W emulsions

Peroxide value (PV) and tocopherol analyses are performed on lipid extracts. Thus, the lipids were extracted from the emulsions prior to these analyses according to the method described by Bligh and Dyer [16] using a reduced amount of solvent [17]. For each sample code two lipid extractions were performed (n=2).

Tocopherols

Lipid extracts were evaporated under nitrogen, re-dissolved in heptane and analyzed by HPLC (Agilent 1100 Series, Agilent Technology, Palo Alto, CA, USA) according to the AOCS Official Method Ce 8-89 [13]. A silica column (Waters (Dublin, Ireland), 150mm, 4.6mm, 3μm silica film) was used for separation of the tocopherol homologues. This analysis was performed in duplicate on each lipid extract and results reported as μg tocopherol / g emulsion.

Primary oxidation products: Peroxide value (PV)

PVs in the lipid extracts were determined by colorimetric method based on formation of an iron-thiocyanate complex. The colored complex was measured on a spectrophotometer at 500 nm (Shimadzu UV1800, Shimadzu Scientific Instruments, Columbia, MD, USA) [18]. The analysis was performed in duplicate and reported as meq peroxides / kg oil.

Secondary oxidation products: Volatiles

Volatiles were released from the ~~o/w~~O/W emulsion using dynamic headspace (45°C for 30 min, nitrogen flow of 150 mL/min). Volatiles were then collected and trapped on Tenax GR packed tubes. To avoid foam and thus water on the tubes, 4 mL of antifoam (Synperonic, conc. 8 g /L water) was added to each sample prior to the collection. Trapped volatiles were desorbed using an automatic thermal desorber (ATD-400, Perkin Elmer, Waltham, MA, USA). The transfer line of the ATD was connected to a gas chromatograph (Agilent 5890, Palo Alto, CA, USA) with a mass selective detector (HP 5972). Volatiles were separated on a DB1701 column (30m x ID 0.25mm x 1μm film thickness, J&W Scientific, Folsom, CA, USA). The initial temperature of the oven was 45°C, which was kept for 5 minutes and then gradually increased as follows: 45-55°C 1.5°C/min, 55-90°C 2.5°C/min, 90-220°C 12°C/min and kept at 220°C for 4 minutes. Calibration curves prepared from external standards was used for quantification of different volatiles. In experiment 1 (Table 1), different concentrations of external standard solutions were prepared and 1 µL was placed in Tenax tubes and analyzed. In experiment 2 (Table 1), different concentrations of external standard solutions were prepared and added to fresh emulsion without antioxidant. Volatiles were collected in the same way as for samples. The 2,4-heptadienal external standard appears as two peaks in the chromotogramme, these peaks are termed A and B. Each sample code was analyzed in triplicate (n=3) and results reported as ng volatile / g emulsion.

Partitioning

Partitioning of caffeic acid and the different alkyl caffeates in buffer/oil, emulsifier/buffer and in ~~o/w~~O/W emulsion was measured according to the method described by Schwarz et al. [19] with modifications as described elsewhere. With this method it is assumed that the partitioning of the antioxidants, equilibra reached, is n~~’~~ot disrupted by centrifugation. For further details refer to Sørensen et al. [20].

In short, the concentration of antioxidants was measured in the separated aqueous phase of 3 different systems: buffer / oil (FO or FO/RO), buffer / emulsifier (Citrem or Tween80) and 5% ~~o/w~~O/W emulsions. Antioxidants were dissolved in methanol and added in a concentration of 100 µM. Separation of the aqueous phase was carried out 24 h after production of the different systems.

Determination of concentration of added antioxidant

Caffeic acid and alkyl caffeates in the aqueous phases were analyzed by HPLC (Agilent 1100 Series, Agilent Technology, CA, USA) with a C18 Thermo Hypersil® ODS (250x4.6 mm, 5μ) column and using a gradient elution at a flow rate of 1 mL/min. Solvent A was 3 mM phosphoric acid and solvent B was methanol. Gradient condition: 0-30 min 0-100% B, 30-40 min 100% B and 40-45 min 100-0% B. Injection volume was 20 µL. Caffeic acid and different alkyl caffeates were quantified by calibration curves of these compounds dissolved in methanol.

Conjugated autoxidizable triene (CAT) Assay

Stock solutions of the different compounds: caffeic acid, caffeates and trolox were prepared in methanol. The CAT assay was performed as described in Sørensen et al. [8] with a modification. This assay was performed with non-stripped tung oil, hence, the assay contained tocopherol and the evaluated antioxidant in a mixture. Each microplate well contained a microemulsion with the following composition: 115 µM tung oil, 17 µM Brij 35, 1 mM AAPH and antioxidant (caffeic acid, caffeates or Trolox) in various concentrations. The progress of lipid oxidation was followed by measuring the decrease in absorbance at 273 nm. Each antioxidant concentration was measured in triplicate on the plate and via independent measurements (two different microplates), n = 6. Results were expressed as CAT value (mean ± SD). This method was developed by Laguerre et al. [21]. For further details about the calculations refer to Laguerre et al. [4, 21].

Data treatment

Statistics. The results obtained were analyzed using one- and two-way ANOVA (GraphPad Prism, Version 4.01, GraphPad Software Inc). Bonferroni multiple comparison post-test was used to determine significant differences between samples or storage times. The significance level applied was 95% ($p < 0.05$). When a sSignificant difference was observed between samples, it is denoted with different superscripts (i.e. a, b, c ect.).

Inhibition percentages. Since different oil and emulsifiers can influence amount of lipid oxidation, inhibition percentages were used to compare the efficacy of the antioxidants in the different emulsion systems. The antioxidants inhibition percentages were calculated according to the following equation:

$$Inhibition\ [\%] = \left(\frac{Emulsion_{Control} - Emulsion_{Antioxidant}}{Emulsion_{Control}}\right) x\ 100$$

$Emulsion_{Control}$ and $Emulsion_{Antioxidant}$ are emulsions without antioxidant and emulsion with antioxidant added, respectively. The antioxidant has a prooxidative effect if the inhibition < -5% and an antioxidative effect > 5%. The antioxidative effect can be categorized as weak (5-20%), intermediate (20-50%) or strong (> 50%) effects [22].

Multivariate data analysis, Principal Component Analysis (PCA). Inhibition percentages calculated from results obtained from PV, volatiles and tocopherol analysis were subjected to PCA using Unscrambler version X10.3 (Camo, Oslo, Norway). The PCA model was built on inhibition percentages calculated on the average of the measured data. Full cross validation was used to validate the model. A PCA allows detection of similarities and dissimilarities between the different samples in a score plot, whereas correlations between the measured variables are visualized in a loadings plot. Connecting these plots in this case shows the degree of oxidation between the different samples.

Results

Physical stability of the emulsions

The physical stability of the emulsions was evaluated by following the oil droplet size (D3,2 (surface weighted mean)) over time. With Citrem as emulsifier (Exp. 1), the droplet size was between 101 – 114 nm for the different Citrem stabilized emulsions (data not shown). The emulsion prepared with Tween as emulsifier (Exp. 2), non-stripped FO/RO and stripped FO/RO had droplet sizes between 124 – 131 nm and 126 - 131 nm, respectively (data not shown). Emulsions produced on the homogenizer (Exp. 1), resulted in slightly smaller oil droplets than emulsions produced on the microfluidizer (Exp. 2). The small differences in oil droplet sizes, however significant, for Citrem and Tween stabilized emulsions is suggested to be due to the different emulsification equipment applied, the different emulsifiers used or a combination. Changes in droplet size during storage were minor, but significant. The changes ranged from -6 nm to +2 nm in size. Since, the oil droplets mainly had a slight decrease in size during storage, the changes is suggested to be due to day to day instrumental variation. Hence, all emulsions were physically stable during the whole storage time, 42 hours, 6 and 15 days, respectively.

Partitioning of caffeic acid and caffeates

The concentration of caffeic acid (CA) and caffeates (CA C1 – CA C12) were determined in the aqueous phase of three different systems to mimic the applied emulsion systems. Hence, the two different emulsifiers and non-stripped and stripped oil was applied. The results obtained are presented in Table 2. In general, the partitioning of caffeic acid and caffeates in the aqueous phase decreased with increased degree of lipophilization until chain length C4 after which the caffeates in most cases could not be detected in the aqueous phase. In the case of the buffer/Tween system octyl- (C8) and dodecyl caffeates (C12) were detected in the aqueous phase and the concentrations were not significantly different from that of butyl caffeate (C4). The concentration of caffeic acid and methyl caffeate in the aqueous phase was significantly lower when Tween was applied as emulsifier than when Citrem was applied. Moreover, the concentration of butyl caffeate tended to be lower (not significant) in the aqueous phase with Tween as emulsifier than with Citrem as also observed for caffeic acid and methyl caffeate. These partitioning results indicated interactions between antioxidants and emulsifier to a higher extent with Tween than Citrem, thus, resulting in a higher concentration of antioxidant at the interface with Tween than Citrem as emulsifier. In addition, the results indicated that there was a tendency~~, however, not significant,~~ to a lower concentration of caffeic acid and methyl caffeate in the aqueous phase of the non-stripped Tween 80 emulsion, i.e. when endogenous tocopherol was present~~, h~~. However, the differences were not significant (Table 2).

Efficacy of caffeic acid and caffeates in emulsions

The antioxidant efficacy of caffeic acid and caffeates were evaluated in the three different emulsions shown in Table 1 and in a modified CAT assay (modification: non-stripped tung oil applied) to investigate the effect of the presence of endogenous tocopherols in this assay. The efficacy of caffeic acid and caffeates was evaluated from the measured lipid oxidation during storage. Lipid oxidation was followed by measuring PV (primary oxidation product) and volatiles (secondary oxidation products). Volatile oxidation products that increased during storage were identified and quantified. The quantified volatiles were markers of lipid oxidation products, most of them originated from oxidation of n-3 PUFAs.

Citrem stabilized emulsions with endogenous tocopherols present. The PV in this experiment increased to 20-35 meq. peroxid~~a~~es / kg oil depending on the antioxidant treatment during the 15 days of storage (data not shown). The concentration of volatiles after 15 days of storage dependended upon the specific volatile quantified and the antioxidant applied (1-penten-3-one 20-60 ng / g emulsion, 1-penten-3-ol 100-300 ng / g emulsion, hexanal 70-150 ng / g emulsion, 4-heptenal 10-25 ng / g emulsion, 2,4-heptadienal 4000-6000 ng / g emulsion and nonanal 30-70 ng / g emulsion).

A PCA of the PVs, α-tocopherol and volatile compounds measured during storage explained 71% of the variation in the obtained results by the first two principal components (PCs), Figure 1. The 3 other tocopherols (β-, δ-, and γ-tocopherols) were excluded from the PCA model due to no or minor changes during storage in the different emulsions. Figure 1A shows the correlation loadings i.e. graphical mapping of the measured variables. Generally, all the volatiles were located in quadrant 1 (top-right part) and 4 (bottom-right part), PVs in quadrant 2 (top-left part) closer to PC 2 and α-tocopherols in quadrant 2 and 3 (bottom-left part) a long PC 1. Thus, the first PC clearly described lipid oxidation with tocopherol in the left side and volatiles in the right side. Comparing Figure 1A with 1B, the scores, reveals the differences in efficacy between caffeic acid and caffeates. Butyl caffeate was prooxidative due to its location in the 4th quadrant i.e. high concentration of many of the volatile compounds. Octyl caffeate also acted as a prooxidant, however, not as strong as butyl caffeate. Furthermore, hexadecyl caffeate's location in first quadrant, but close to PC 2, indicates a slight prooxidative effect. Dodecyl and eicosyl caffeates seemed to have no effect due to their proximity to the control emulsion in the 2nd quadrant. The location of caffeic acid in the 3 quadrant of the scores plot away from the volatile compounds (1 and 4 quadrant) indicates that this antioxidant was the most efficient followed by methyl caffeate. However, caffeic acid and methyl caffeate were not strong antioxidants, since some of the measured volatiles were present in higher concentration in these emulsions than in the control emulsion (Table 3, Inhibition percentages, Raw data not shown).

Tween stabilized emulsions with endogenous tocopherols present. In the Tween stabilized emulsion with endogenous tocopherols present, the PV increased to 5-30 meq. peroxides / kg oil depending on the antioxidant treatment during the 6 days of storage. The concentration of volatiles after 6 days of storage dependent upon the specific volatile quantified and the antioxidant applied (1-penten-3-one 2-10 ng / g emulsion, 1-penten-3-ol 5-30 ng / g emulsion and 2,4-heptadienal 50-350 ng / g emulsion).

A PCA of the PVs, α-tocopherol and volatile compounds (1-penten-3-one, 1-penten-3-ol and 2,4-heptadienal) measured during storage explained 82% of the variation in the obtained results by the first two PCs (Figure 2). Similar to the other PCA model on Citrem stabilized emulsions, the 3 other tocopherol homologues (β-, δ-, and γ-tocopherols) were excluded from the PCA model due to no or minor changes during storage in the different emulsions. The PV and all the volatiles were located to the left in the plot of the correlation loadings (Figure 2A). Tocopherols were located opposite to the PV and volatiles. From the correlation loadings plot it is clear that the first PC describe lipid oxidation with tocopherol in the right side and volatiles in the left side, thus, increased lipid oxidation moving from the right to the left in the plot. Scores plot (Figure 2B) reveals differences in efficacy between caffeic acid and caffeates in the tween stabilized emulsions. The control emulsion was located to the left side of the PC 2 axis in the scores plot and all emulsions with antioxidant added were located to the right of the PC 2 axis except emulsion with butyl caffeate added (Figure 2B). In connection with correlation loadings plot, this indicates that all the emulsions with antioxidant added acted as antioxidant in the tween stabilized emulsions. As described above, butyl caffeate was located opposite to the PC 2 axis compared to the other emulsions with antioxidant added, this is explained by the higher amount of 2,4-heptadienal at day 6 in this emulsion (Figure

2). Butyl caffeate worked as antioxidant for all other oxidation parameters measured; however, it was the least efficient due to higher amount of the measured oxidation parameters compared to the other antioxidant applied (Table 3). Moreover, the PCA model reveals that caffeic acid without esterification was more efficient in tween stabilized emulsions followed by methyl caffeate (short chain esterification) due to their location most far away from the oxidation parameters measured. Raw data supported the PCA model.

Tween80 stabilized emulsions without endogenous tocopherols present. In the Tween stabilized emulsion with endogenous tocopherols present, the PV increased ~~to~~ from ~~0~~8~~-~~ to 14 meq. peroxid~~a~~es / kg oil without antioxidant added~~depending on the antioxidant treatment~~ during the 42 hours of storage; whereas, the PV increased from 2~~-~~to 8 and 1~~-~~ to 4 meq. ~~P~~peroxides / kg oil with caffeic acid and eicosyl caffeates, respectively~~.~~ The other antioxidant treatments resulted in no increase in PV during storage. ~~-~~The concentration of volatiles after 42 hours of storage dependent upon the specific volatile quantified and the antioxidant applied (1-penten-3-one 0-3 ng / g emulsion, 1-penten-3-ol 0-9 ng / g emulsion and 2,4-heptadienal 2-90 ng / g emulsion), which was much lower than in the Citrem stabilized emulsions.

A PCA of the PVs and volatile compounds (1-penten-3-one, 1-penten-3-ol~~, 4-heptenal~~ and 2,4-heptadienal) measured during storage explained 83% of the variation in the obtained results by the first to principal components (PCs), Figure 3. All the volatiles were located in quadrant 1 (top-right part) and 4 (bottom-right part). The first PC clearly describes lipid oxidation (right side) versus no lipid oxidation (left side), whereas, PC 2 describes the development of lipid oxidation over time, with PV and volatiles in the beginning of the storage period located in the top of the plot and in the bottom of the plot after 42 days~~here increasing oxidation has decreasing PC 2 values~~ (Figure 3A). Comparing Figure 3A with 3B, the scores plot, it is observed that control emulsion and emulsion with caffeic acid is located in the same side as the oxidation parameters measured. Thus, caffeic acid is acting as a prooxidant in Tween stabilized emulsions without endogenous tocopherol present. Esterification of caffeic acid in Tween stabilized emulsion without tocopherols improved its antioxidative properties, since all caffeates evaluated were acting as antioxidants. However, it seems like caffeic acid esterified with C20 was slightly less efficient than the other esters evaluated (Table 3). Raw data supports the observation from the PCA model (data not shown).

Comparison of the influence of emulsifier type - Citrem versus Tween80 stabilized emulsions. Table 3 shows calculated inhibition percentages for caffeic acid and caffeates. It is clear that the emulsifier impacted the efficacy of the antioxidants added. In Citrem stabilized emulsions, caffeic acid and methyl caffeate were the only ones acting as antioxidants. Their antioxidative effect in this model emulsion was weak to intermediate and they even promoted the formation of certain volatiles. In contrast, caffeic acid and all the evaluated caffeates in Tween stabilized emulsions acted as antioxidants. Caffeic acid was the strongest antioxidant followed by methyl caffeate.

Comparison of the influence of the presence of endogenous tocopherols. Calculated inhibition percentages for selected oxidation variables measured in Tween stabilized emulsions with and without endogenous tocopherols are presented in Table 3. A clear difference in the efficacy of antioxidants was that caffeic acid acted as a strong antioxidant when tocopherols were present, whereas, it acted as intermediate to strong prooxidant without tocopherols in this model emulsion. In addition, the caffeates acted as stronger antioxidants without tocopherols compared to the same model emulsion with tocopherols present (Table 3). Caffeates with chain lengths between C1 and C18 were all strong antioxidants, whereas the antioxidant efficacy was decreased when the chain length was increased to C20.

Antioxidant efficacy in a modified CAT assay. The CAT assay is an assay developed to measure the efficacy of antioxidants in a micro emulsion system without endogenous tocopherols present.

The assay was slightly modified to investigate the efficacy of caffeic acid and caffeates in the presence of endogenous tocopherols. The results are shown in Figure 4 together with results obtained earlier with an unmodified CAT assay [8]. When tocopherol was present the antioxidative efficacies of caffeic acid, methyl-, butyl-, octyl- and dodecyl caffeates were not significantly different. This finding is different from earlier results obtained without the presence of tocopherols, where octyl- and dodecyl caffeates exerted a significantly higher efficacy than caffeic acid and the other caffeates evaluated. Furthermore, the efficacy of octyl- and dodecyl caffeates without tocopherols present was also significantly higher than when tocopherols were present.

Discussion

The results showed - as hypothesized - that partitioning of the antioxidants was affected by the type of emulsifier and the presence of endogenous tocopherols. Earlier measurements of the radical scavenging effect (DPPH assay) of caffeic acid and caffeates showed no differences between the caffeic acid and the different saturated unbranched alkyl esters [8]. In spite of that the antioxidative effect of caffeic acid and caffeates was different in the different emulsion systems. However, the activity can differ due to the emulsion composition.

Impact of emulsifier on the efficacy of caffeic acid and caffeates. In the present study, caffeic acid and caffeates were more efficient antioxidants in Tween stabilized emulsions than in Citrem stabilized emulsions. This partly supports earlier findings with the same emulsifiers and caffeic acid in 10% ~~o/w~~O/W emulsions, where caffeic acid in Citrem stabilized emulsions promoted the formation of volatiles and no effect on lipid oxidation or slightly antioxidative effect of caffeic acid was observed in Tween stabilized emulsions [23]. Besides different oil concentration in the previous and current studies, antioxidant concentration (5.5 fold ~~higher~~ lower in ~~the present~~ this study) and oil / emulsifier ratio were also different, which can have an influence on the differences observed in these studies. Independently of emulsifier type, caffeic acid was performing better than the caffeates. Contrary, the effect of the caffeates was affected by the emulsifier applied. To our knowledge ~~O~~only few studies related to the antioxidative effect of caffeic acid and~~,~~ caffeates in emulsions have been published [9, 24, 25]. These studies did not compare the effect of the emulsifier type applied. However, one of them compared the effect of caffeates in mayonnaise and milk, where not only the emulsifier type is different but the entire emulsion system. The efficiency of the caffeates was affected by the type of emulsion system [9]. Results obtained in the present study also demonstrated that changing the emulsifier affected the antioxidative effect and rank order of caffeates. Experiments performed with gallic acid and ethyl gallate have also shown that changing emulsifier affects the partitioning of the antioxidants and the resulting antioxidant activity measured in emulsion systems [26]. The different emulsifier evaluated was SDS, CTAB, Brij58 and PHLC, and their partitioning study revealed increased solubility effect of the emulsifiers in the following order: PHLC < SDS < Brij58 < CTAB. The antioxidant activity of gallic acid and ethyl gallate based on the formation of hydroperoxides and hexanal increased in the following order: CTAB (no activity measured) < Brij58 < PHLC < SDS. Gallic acid only showed antioxidant activity with PHLC stabilized emulsions. This was a reverse order compared to the partitioning measured. Hence, it was suggested that the increased partitioning into the emulsifier layer and lipid counteract the hydrogen-donating ability, and lower the activity of the antioxidants [246]. Moreover, Pekkarinen et al. [257] evaluated antioxidative effect and partitioning of phenolics in different systems. Interaction between caffeic acid and Tween 20 differed from other phenolics such as vanillic acid, ferulic acid and sinapic acid evaluated, since Tween 20 exhibited higher solubilisation capacity for caffeic acid than for other phenolic acids. Additionally, Pekkarinen et al. [257] concluded that these antioxidant-emulsifier interactions have a strong influence on the partitioning of antioxidants. The partitioning results obtained in this study confirmed that Citrem and Tween as emulsifiers results in differences in the partitioning of caffeic acid and caffeates. Less caffeic acid and caffeates were present in the aqueous phase when Tween was applied. This clearly demonstrated stronger antioxidant-emulsifier interaction with Tween compared to Citrem. Moreover, Citrem is an anionic emulsifier, thus, the interface is negatively charged and will repel negatively charged antioxidants i.e. caffeic acid, which could explain why caffeic acid interacted less with Citrem than with Tween. An explanation for the stronger interactions with Tween may be the molecular structure of the emulsifiers, since Tween is a larger and more bulky molecule than Citrem; however, this has to be further evaluated. Furthermore, Schwarz et al. [19, 268, 279] evaluated partitioning of different antioxidants in dispersed lipid systems with different emulsifiers.

Significant differences were observed in partitioning of the antioxidants between phases, both as a function of pH and emulsifier type and concentration. It was concluded from the results that determination of antioxidant partitioning may be an important tool to select antioxidants structurally designed to localize at the surfaces [19], however, the partitioning of the antioxidants cannot alone explain the measured antioxidant activity in emulsions [2~~7~~9]. Results from another study by Schwarz et al. [~~28~~30] evaluating antioxidant activity of antioxidants with different lipophilicity in bulk oil, ~~o/w~~O/W and ~~w/o~~W/O emulsions with different emulsifiers led to the assumption that differences in antioxidant activity for the same emulsion type might be additionally influenced by interaction with the emulsifier dominating the interfaces in the emulsion system [~~28~~30]. The obtained results for the two emulsifiers together with the partitioning study may also here lead to the assumption that emulsifier-antioxidant interactions (e.g. hydrogen b~~i~~onding) affected the antioxidant activity of the caffeates. However, the type of interactions and the impact of antioxidant-emulsifier interactions on the radical scavenging activity have to be studied ~~more~~ in more details to make further conclusions.

Impact of endogenous tocopherols on the efficacy of caffeic acid and caffeates. The presence of endogenous tocopherol not only changed the antioxidant activity of caffeic acid and caffeates in both the storage experiment and in the CAT assay, but also their partitioning in the emulsion system. A tendency to less caffeic acid and caffeates (C1, C4 and C12) present in the aqueous phase with endogenous tocopherol in the emulsion system was observed. This may indicate some interactions between tocopherol and caffeic acid / caffeates both for the antioxidative effect and localization in the emulsion system. The use of a combination of antioxidants to produce synergistic interaction has been reported earlier e.g. tocopherol regeneration by ascorbic acid, polyphenols and flavonoids [~~29~~31-3~~2~~4]. Pan~~a~~ya et al. [3~~2~~4] carried out the only study investigating interactions between tocopherol and a phenol (rosmarinic acid) and its alkyl esters (rosmarinates, C4, C12 and

C20) in Tween20 stabilized emulsions. Rosmarinic acid exhibited strongest synergistic interaction with tocopherol, and C4 and C12 esters exhibited small synergistic interaction. An antagonistic interaction was observed with C20 ester and tocopherol. Thus, the more hydrophilic rosmarinic acid exhibited more interactions with the tocopheryl radical than the esters. In the present study, the emulsion with the more hydrophilic caffeic acid exhibited better oxidative stability than the emulsions with the esters (more hydrophobic antioxidants) when tocopherol was present as also observed with rosmarinic acid and rosmarinates. Actually, caffeic acid turned from being prooxidative without tocopherol present to being the most efficient antioxidant with endogenous tocopherol present in Tween stabilized emulsion. In emulsions, the majority of the emulsifier is accumulated at the oil-water interface. However, a part of the emulsifier is not associated with the oil-water interface if the emulsifier concentration is above the CMC (critical micellar concentration, CMC Tween80 13-15 mg/L, Sigma) and will form micelles in the aqueous phase. In this study, the concentration of Tween was much higher than CMC (10 g/L). The decreased antioxidant efficiency of the different caffeates compared with caffeic acid is suggested to be due to the solubilisation of caffeates in Tween micelles, thus, localized away from the interface unable to inhibit lipid oxidation. Although, micelles are not isolated structures, they compromise structures that are in dynamic equilibrium with other structures in the emulsion system. This means that components can be exchanged between the different structures i.e. between micelles and emulsion droplets [35, 36]. Thus, more studies are needed to further elucidate the differences observed in partitioning and antioxidant effect in the presence of endogenous tocopherols.

Additionally, it is assumed that caffeic acid is located in close proximity to the interface where it regenerates tocopherol at the interface in spite of the repelling effect of two negatively charged compounds (Citrem and caffeic acid). The proposed partial location of tocopherol at the interface is supported by Jacobsen et al. [37] who reported that ca. 6 % of the alpha-tocopherol present in

518 mayonnaise was located at the interface whereas the remaining tocopherol was located in the oil
519 phase. Therefore, lipophilization is not needed to improve the oxidative stability of these emulsions,
520 due to tocopherols location at the interface.

521 ***Antioxidant hypotheses and efficacies of caffeic acid and caffeates.*** The polar paradox was not
522 confirmed in this study since caffeic acid was more efficient as antioxidant than caffeates in Citrem
523 stabilized emulsions when endogenous tocopherol was present. In Tween stabilized emulsions with
524 endogenous tocopherol caffeic acid followed by methyl caffeate were the most efficient
525 antioxidants, whereas, caffeic acid acted as a prooxidant when tocopherol was not present. No cut-
526 off effect was observed for the lipophilized caffeic acid, since the most efficient antioxidant in both
527 Citrem and Tween stabilized emulsions when tocopherol was present was C0 (caffeic acid). A
528 similar finding was observed for the CAT assay with endogenous tocopherol present. Without
529 endogenous tocopherol present, the caffeates were most efficient antioxidants in Tween stabilized
530 emulsions, a cut-off effect was found at C16. However, the efficiency of C20 was still an
531 intermediate to strong antioxidant. The CAT assay showed a cut-off effect at around C8 and C12
532 with no endogenous tocopherol present. The partitioning experiment clearly showed an effect of the
533 chain length, with less antioxidant present in the aqueous phase with increasing antioxidant
534 lipophilicity. Pekkarinen et al. [247] observed that the proportion of antioxidant solubilized in the
535 lipid phase and particularly in the interface did not necessarily reflect the efficiency of the
536 antioxidant. It was assumed from their evaluation of antioxidant activity and partitioning that
537 specific interactions of the antioxidant with other compounds e.g. emulsifiers, and intermolecular
538 hydrogen bonds may play an important role in reducing antioxidant activity. Furthermore,
539 antioxidant-emulsifier interaction has a strong influence on partitioning of the antioxidant. It is
540 suggested that the caffeates interacts with the emulsifier in form of micelles when endogenous
541 tocopherols are present, resulting in reduced or no antioxidant activity. When endogenous

tocopherols are not present the caffeates is more likely to be solubilized at the interface instead of the tocopherols. However, this has to be evaluated more in depth to conclude further on these differences in partitioning and antioxidant activity with the impact of endogenous tocopherols.

Conclusions. Partitioning and antioxidant activity of caffeic acid and caffeates were influenced both by the emulsifier type and the presence of endogenous tocopherols. Thus, this study clearly demonstrated different emulsifier-antioxidant and antioxidant-antioxidant interactions that affected the efficacy of the evaluated caffeic acid and caffeates as antioxidant in emulsions. The hypotheses about antioxidant in emulsions are based on simple emulsions systems without the presence of tocopherols. However, the impact of the presence of tocopherols on the efficacy of other antioxidants is important since most food systems contain tocopherol.

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Figure legends

Figure 1 PCA build on results obtained from PV, vol~~t~~atiles (1-penten-3-one, 1-penten-3-ol, 4-heptenal, 2,4-heptadienal, hexanal and nonanal) and α-tocopherols measured on Citrem stabilized emulsions during storage (15 days) using full cross validation. A) Correlation loadings and B) Scores plot. Abbreviations for sample codes refer to Table 1.

Figure 2 PCA build on results obtained from PV, vol~~t~~atiles (1-penten-3-one, 1-penten-3-ol and 2,4-heptadienal) and α-tocopherols measured on ~~tween~~ Tween80 stabilized emulsions during storage (6 days) using full cross validation. A) Correlation loadings and B) Scores plot. Abbreviations for sample codes refer to Table 1.

Figure 3 PCA build on results obtained from PV and vol~~t~~atiles (1-penten-3-one, 1-penten-3-ol and 2,4-heptadienal) measured on ~~tween~~ Tween80 stabilized emulsions during storage (42 hours) using full cross validation. A) Correlation loadings and B) Scores plot. Abbreviations for sample codes refer to Table 1.

Figure 4 CAT Value of caffeic acid and caffeates (C1-C16) measured in the concentration range of 0.5 – 2 μM. ● CAT Values determined without endogenous tocopherols (normal condition for the CAT assay, published in Sørensen et al. [8]) and ○ CAT Values determined with endogenous tocopherols (modified CAT assay).

Table 1 Experimental design of experiment 1 and 2.

Experiment	Sample code	Emulsifier	Oil	Antioxidant
	C_Con	Citrem	FO	*No antioxidant*
	C_CA C0	Citrem	FO	*Caffeic acid*
E	C_CA C1	Citrem	FO	*Methyl caffeate*
X	C_ CA C4	Citrem	FO	*Butyl caffeate*
P	C_CA C8	Citrem	FO	*Octyl caffeate*
	C_CA C12	Citrem	FO	*Dodecyl caffeate*
1	C_CA C16	Citrem	FO	*Hexadecyl caffeate*
	C_ CA C20	Citrem	FO	*Eicosyl caffeate*
	T_Con	Tween80	FO/RO	*No antioxidant*
	T_CA C0	Tween80	FO/RO	*Caffeic acid*
	T_ CA C1	Tween80	FO/RO	*Methyl caffeate*
	T_ CA C4	Tween80	FO/RO	*Butyl caffeate*
E	T_ CA C8	Tween80	FO/RO	*Octyl caffeate*
X	T_CA C12	Tween80	FO/RO	*Dodecyl caffeate*
P	T_CA C16	Tween80	FO/RO	*Hexadecyl caffeate*
	TS_Con	Tween80	S FO/RO	*No antioxidant*
2	TS_CA C0	Tween80	S FO/RO	*Caffeic acid*
	TS_CA C1	Tween80	S FO/RO	*Methyl caffeate*
	TS_ CA C4	Tween80	S FO/RO	*Butyl caffeate*
	TS_CA C8	Tween80	S FO/RO	*Octyl caffeate*
	TS_CA C12	Tween80	S FO/RO	*Dodecyl caffeate*
	TS_CA C16	Tween80	S FO/RO	*Hexadecyl caffeate*
	TS_CA C20	Tween80	S FO/RO	*Eicosyl caffeate*

Abbreviations: FO Fish oil, FO/RO Fish oil and rapeseed oil (1:1, w/w) and S FO/RO Stripped fish oil and rapeseed oil (1:1, w/w)

Table 2 Concentration [µM] of caffeic acid and caffeates (Methyl, Butyl, Octyl and Dodecyl) measured in the aqueous phase of different systems: 95% Buffer / 5% Oil, 99% Buffer / 1% Emulsifier and Emulsion (5% Oil, 1% Emulsifier and 94% Buffer). Citrem and Tween80 were applied as emulsifier. Both non-stripped and stripped FO/RO was evaluated with Tween80 as emulsifier.

Antioxidant	Citrem and non-stripped oil			Tween80 and non-stripped oil			Tween80 and stripped oil	
	Buffer / Oil	**Buffer / Emulsifier**	**Emulsion**	**Buffer / Oil**	**Buffer / Emulsifier**	**Emulsion**	**Buffer / Oil**	**Emulsion**
CA C0	101 ± 3.1^{a,b,x}	91.5 ± 3.6^{b,x}	91.5 ± 7.1^{b,x}	93.7 ± 11.4^{b,x}	75.4 ± 7.0^{c,x}	74.0 ± 4.3^{c,x}	111 ± 2.6^{a,x}	78.4 ± 10.1^{c,x}
CA C1	86.2 ± 0.9^{a,y}	53.3 ± 3.0^{b,y}	43.8 ± 8.2^{b,y}	82.8 ± 1.9^{a,x}	15.4 ± 3.3^{c,y}	10.1 ± 2.5^{c,y}	91.1 ± 2.6^{a,y}	15.3 ± 3.1^{c,y}
CA C4	11.4 ± 0.4^{a,b,z}	4.09 ± 0.6^{a,b,c,z}	3.39 ± 0.5^{b,c,z}	11.3 ± 0.7^{a,b,y}	2.10 ± 0.3^{c,z}	< detection	13.0 ± 1.1^{a,z}	< detection
CA C8	< detection	< detection	< detection	< detection	2.60 ± 3.6^{z}	< detection	< detection	< detection
CA C12	< detection	< detection	< detection	2.00 ± 0.6^{b,z}	1.10 ± 0.4^{z}	< detection	9.70 ± 4.2^{a,z}	1.10 ± 0.2^{b,y}

Different letters in superscript indicate significant differences. Significant differences within a row i.e. same antioxidant but different systems are denoted with a,b and c, whereas significant differences within a column i.e. same system but different antioxidant are denoted with x, y and z.

Table 3 Calculated inhibition percentages for selected lipid oxidation parameters (PV, 1-penten-3-ol, 1-penten-3-one and 2,4-heptadienalA) measured at selected time points in the different emulsions. Citrem stabilized emulsions are reported at day 6 and 15, Tween stabilized emulsions with endogenous tocopherols at day 6 and Tween stabilized emulsions without endogenous tocopherols at 42 hours.

AO	Citrem (Day 6)				Tween with tocopherols				Tween without tocoherols			
	PV	1Pen3ol	1Pen3one	2,4HepA	PV	1Pen3ol	1Pen3one	2,4HepA	PV	1Pen3ol	1Pen3one	2,4HepA
CA	50	39	3	44	79	83	86	86	34	-63	-55	-48
C1	32	1	-210	-27	60	60	69	11	89	97	85	89
C4	21	-119	-502	-143	47	61	33	-10	89	99	83	93
C8	-28	-174	-603	-87	49	71	52	-3	88	100	94	94
C12	-53	-130	-515	-13	43	62	55	2	91	102	97	95
C16	-26	-104	-487	-39	44	58	39	4	87	101	96	98
C20	-31	-43	-340	8					66	59	44	45
AO	Citrem (Day 15)											
	PV	1Pen3ol	1Pen3one	2,4HepA								
CA	13	-166	52	13								
C1	31	-123	65	18								
C4	28	-61	-65	-41								
C8	54	-47	-51	-17								
C12	5	-10	-28	5								
C16	-7	3	-21	5								
C20	-5	12	-15	7								

Abbreviation: AO Antioxidant; PV Peroxid~~ae~~ ~~v~~Value; 1Pen3ol 1-Penten-3-ol; 1Pen3one 1-Penten-3-one; 2,4HepA 2,4-HeptadienalA. The 2,4-heptadienal external standard appears as two peaks in the chromotogramme, these peaks are termed A and B (here only 2,4HepA presented).

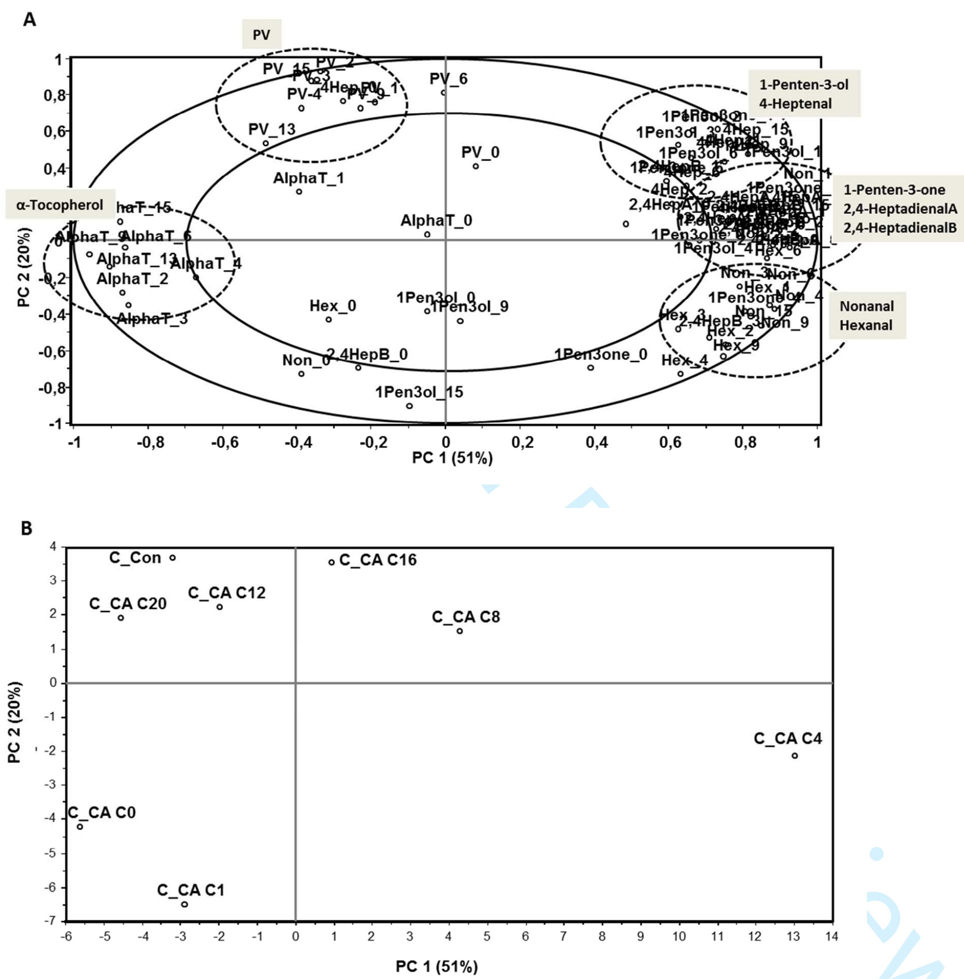
A
PV
1-Penten-3-ol
4-Heptenal
α-Tocopherol
1-Penten-3-one
2,4-HeptadienalA
2,4-HeptadienalB
Nonanal
Hexanal
PV_6
PV_13
PV_0
AlphaT_1
AlphaT_0
AlphaT_4
AlphaT_2
AlphaT_3
Hex_0
1Pen3ol_0
1Pen3ol_9
Non_0
2,4HepB_0
1Pen3one_0
1Pen3ol_15
PC 1 (51%)
PC 2 (20%)
B
C_Con
C_CA C16
C_CA C12
C_CA C20
C_CA C8
C_CA C4
C_CA C0
C_CA C1
PC 1 (51%)
PC 2 (20%)

Figure 1

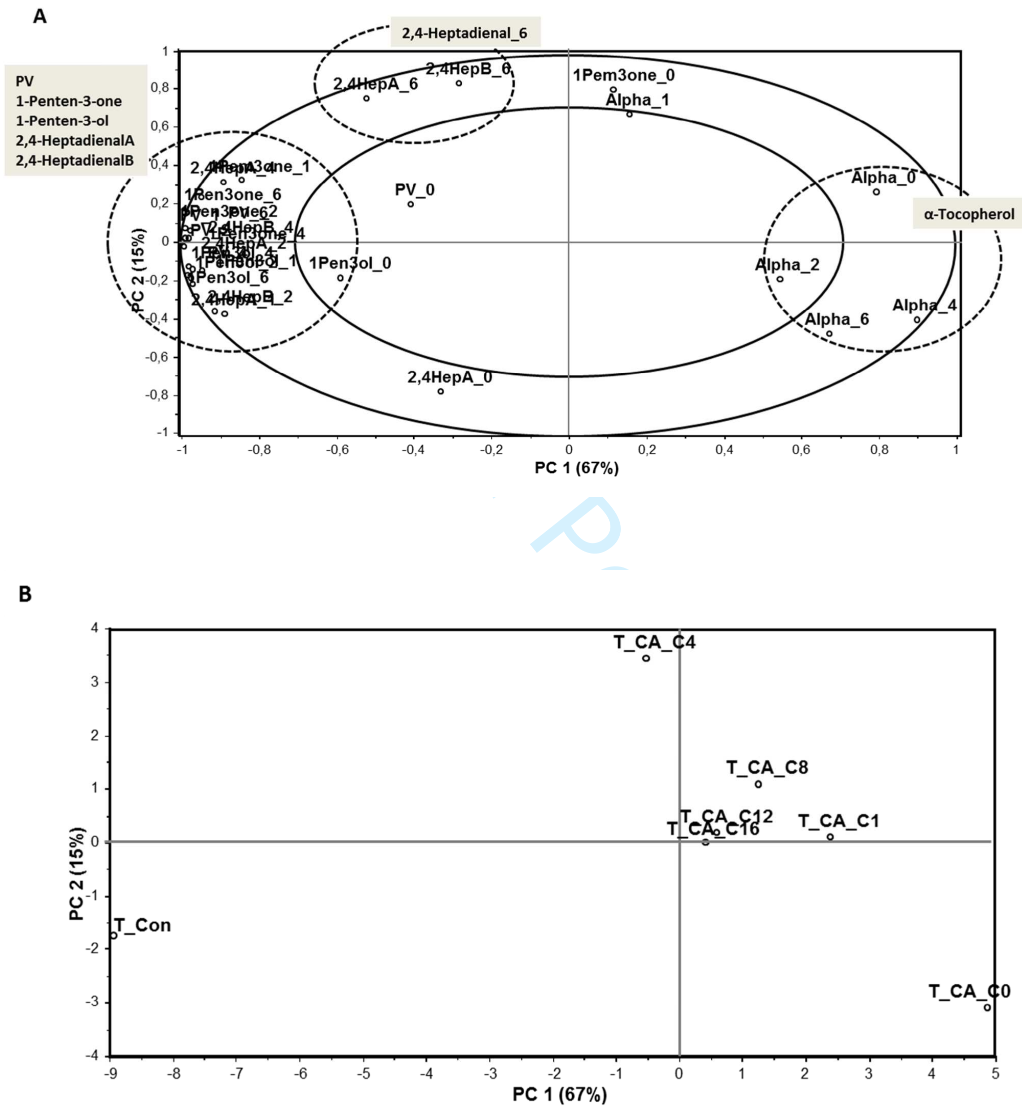
A
2,4-Heptadienal_6
PV
1-Penten-3-one
1-Penten-3-ol
2,4-HeptadienalA
2,4-HeptadienalB
2,4HepA_6
2,4HepB_6
1Pem3one_0
Alpha_1
PV_0
1Pen3ol_0
1Pen3one_6
Pen3ol_6
Alpha_0
α-Tocopherol
Alpha_2
Alpha_6
Alpha_4
2,4HepA_0
PC 2 (15%)
PC 1 (67%)
B
T_CA_C4
T_CA_C8
T_CA_C12
T_CA_C16
T_CA_C1
T_Con
T_CA_C0
PC 2 (15%)
PC 1 (67%)

Figure 2

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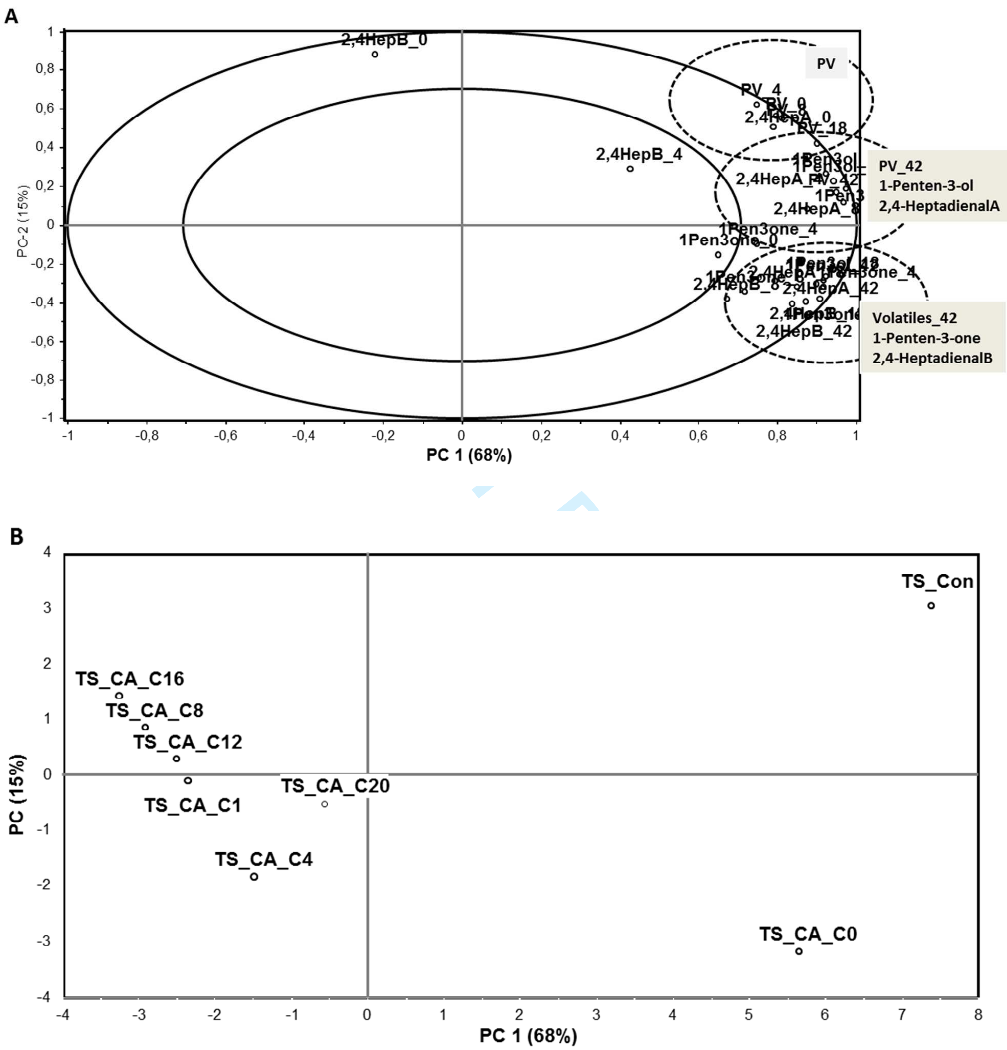
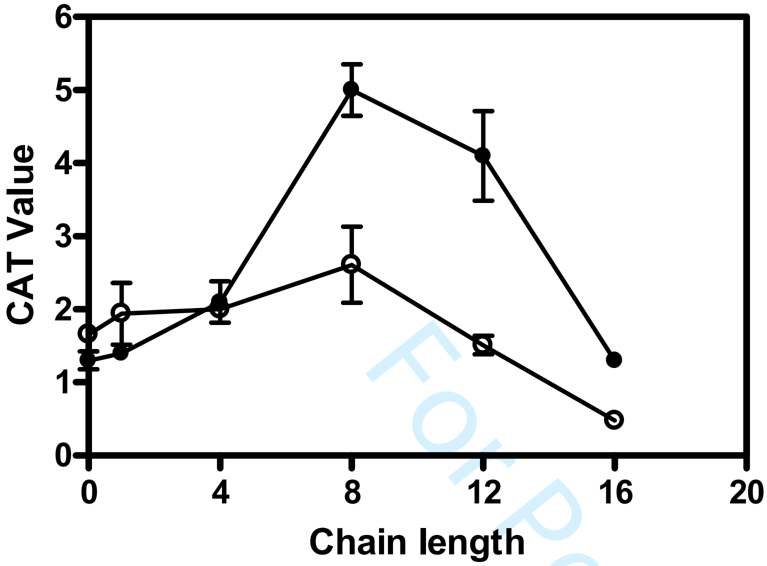

A
2,4HepB_0
2,4HepB_4
PV
PV_4
2,4HepA_0
PV_42
1-Penten-3-ol
2,4-HeptadienalA
1Pen3one_4
1Pen3one_0
2,4HepA_42
2,4HepB_42
Volatiles_42
1-Penten-3-one
2,4-HeptadienalB
PC-2 (15%)
PC 1 (68%)
B
TS_Con
TS_CA_C16
TS_CA_C8
TS_CA_C12
TS_CA_C1
TS_CA_C20
TS_CA_C4
TS_CA_C0
PC (15%)
PC 1 (68%)

Figure 3



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711 **Figure 4**

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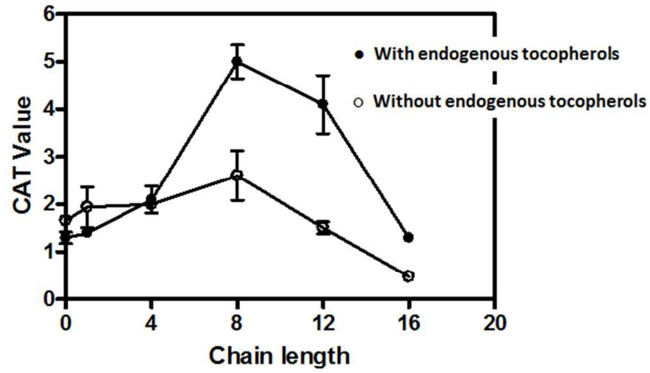
Graphical abstract. CAT Value determined in oil-in-water micro emulsions with caffeic acid and caffeates (100 µM) and with and without endogenous tocopherol and. Lipid oxidation was initiated with the water soluble radical AAPH.



254x142mm (96 x 96 DPI)