



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

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

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

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11 Running title: Effect of emulsifier and antioxidants on lipid oxidation

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

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

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4 **19 Abstract**

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7 20 Antioxidant addition can be one strategy to limit lipid oxidation in emulsions. Research has proven  
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9 21 that an important factor regarding the efficacy of antioxidants is their localization in the emulsion;  
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11 22 however, other factors such as interactions with other components can also have an impact. Thus,  
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13 23 the aim was to evaluate the impact of emulsifiers (Citrem and Tween80) and presence of  
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15 24 endogenous tocopherols on the efficacies of caffeic acid and caffeates (C1-C20) as antioxidants in  
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17 25 emulsions. Lipid oxidation was evaluated during storage and partitioning of caffeic acid and  
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19 26 caffeates was estimated by measuring their concentrations in the aqueous phase.

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22 27 Partitioning of caffeic acid and caffeates was influenced by emulsifier type and presence of  
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24 28 endogenous tocopherols. Caffeic acid was the most efficient antioxidant in Citrem and Tween  
25  
26 29 stabilized emulsions in the presence of endogenous tocopherol. In contrast, for Tween stabilized  
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28 30 emulsions, caffeic acid acted as a prooxidant and the evaluated caffeates acted as strong  
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30 31 antioxidants in the absence of endogenous tocopherol. Thus, when endogenous tocopherol was  
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32 32 present lipophilization of caffeic acid did not increase its efficacy as an antioxidant. It is suggested  
33  
34 33 that the differences observed in antioxidant efficiency with different emulsifiers and with and  
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36 34 without endogenous tocopherols is due to emulsifier-antioxidant interactions and antioxidant-  
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38 35 antioxidant interactions in the emulsions.

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41 36 **Practical application:** Food emulsions contain endogenous tocopherols, thus, the impact of  
42  
43 37 endogenous tocopherols on the efficacy of applied antioxidants is of interest to the industry. So far  
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45 38 the hypotheses about antioxidant in emulsions are based on simple emulsions systems without the  
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47 39 presence of tocopherols. The finding in this study revealed that both emulsifier type and the  
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49 40 presence of endogenous tocopherol had an impact on the efficacy of caffeic acid and caffeates due  
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51 41 to emulsifier-antioxidant and antioxidant-antioxidant interactions. This highlights the importance of  
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42 evaluating the antioxidant in each emulsion system before selecting antioxidants for optimal  
43 protection against lipid oxidation.

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## 46 **Introduction**

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

53 Different strategies can be applied to limit lipid oxidation and thereby improve shelf life of  
54 emulsions [2]. One of them is addition of antioxidants; however, selection of the right antioxidant  
55 or mixture of antioxidants is difficult, since their efficacies are affected by the composition of the  
56 emulsions e.g. their localization and interaction with other components. An enormous amount of  
57 studies have been reported in the literature on antioxidants and their efficacies in model emulsions  
58 and more complex food emulsions. So far, two hypotheses about antioxidant efficacies in  
59 emulsions, namely the polar paradox hypothesis [3] and the cut-off effect [4] have been reported. In  
60 brief, the polar paradox hypothesizes that apolar antioxidants are more efficient in O/W emulsions  
61 than polar antioxidants [3] due to differences in the antioxidants' affinity towards the different  
62 phases [5]. The cut-off effect can be seen as an extension of the polar paradox hypothesis, in which  
63 an optimal degree of lipophilisation for optimal antioxidant activity is observed [4], which is called  
64 critical chain length (CCL). The cut-off effect was observed from results obtained with chlorogenic  
65 acid and rosmarinic acid and their unbranched saturated alkyl esters (chlorogenates and  
66 rosmarinates). The efficacy of antioxidant homologues was related to the partitioning of these  
67 antioxidants in an emulsion system [4, 6]. Based on these observations, it was assumed that when  
68 the lipophilized antioxidants had the CCL they were present in the highest concentration at the oil-  
69 aqueous interface, where lipid oxidation is initiated. In addition, antioxidant homologues with chain

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4 70 length below and above CCL were driven away from the oil-aqueous interface [7]. These  
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6 71 antioxidant hypotheses are based on extensive research in simplified emulsions prepared with  
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8 72 stripped oils, whereas food emulsions contain e.g. endogenous tocopherols. Only few compounds  
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10 73 with different degree of lipophilization have been evaluated in several different emulsion systems.  
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12 74 Caffeic acid and unbranched saturated caffeates have been evaluated both in a model emulsion  
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14 75 (CAT assay) system [8], milk and mayonnaise [9] and CCL seemed to be influenced by the system.  
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16 76 In addition, rosmarinic acid has also been evaluated in different systems; model emulsions and low  
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18 77 moisture food (crackers). In model emulsions, a parabolic relationship between antioxidant efficacy  
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20 78 and hydrophobicity was observed, with the intermediate polarity (8 carbon – 18 carbon chain  
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22 79 length) giving optimum activity [6, 10, 11]. In crackers, a linear relationship between antioxidant  
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24 80 activity and hydrophobicity was observed [12].  
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28 81 Due to formerly obtained results with caffeic acid and caffeates in model emulsion, milk and  
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30 82 mayonnaise [8, 9], it is hypothesized that emulsifier type and the presence of endogenous  
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32 83 tocopherols can affect the partitioning of caffeic acid and caffeates and thereby change their  
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34 84 efficacy in the emulsion systems. Hence, the aim of this study was to evaluate the impact of both  
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36 85 emulsifier and presence of endogenous tocopherols on the efficacies of caffeic acid and caffeates  
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38 86 (C1-C20). Two experiments were carried out as shown in Table 1. In the first experiment, the aim  
39  
40 87 was to evaluate the effect of lipophilization of caffeic acid on its antioxidant efficacy in an O/W  
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42 88 emulsion prepared with unstripped oil and Citrem as emulsifier. The aim of the second experiment  
43  
44 89 was to investigate whether the presence of endogenous tocopherol affected the optimal chain length  
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46 90 of lipophilized caffeic acid when Tween was used as an emulsifier. Moreover, comparison of results  
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48 91 of the two experiments enabled an evaluation of whether the emulsifier type, Citrem vs Tween  
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50 92 affected the CCL in emulsions prepared with unstripped oil.  
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4 93 In both experiments, the partitioning of the antioxidant was estimated by measuring its  
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6 94 concentration in the aqueous phase of the O/W emulsion, a buffer/oil system and a buffer/emulsifier  
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8 95 system to evaluate if emulsifier type and the presence of endogenous tocopherols, affected the  
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10 96 partitioning of caffeic acid and caffeates in the emulsion system.  
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## 98 **Material and Methods**

99 The oil used in this study was fish oil (FO) and a mixture between fish and rapeseed oil (FO/RO,  
100 1:1). The two types of oil were supplied by Maritex A/S (TINE BA, Sortland, Norway). The quality  
101 of the FO was as follows: 0.3 meq. peroxides / kg oil, 250 mg  $\alpha$ -tocopherol / kg, 98 mg  $\gamma$ -  
102 tocopherol / kg and 48 mg  $\delta$ -tocopherol / kg. Fatty acid composition of the fish oil was as follows:  
103 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%,  
104 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,  
105 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%  
106 and 1.8 %, respectively. The quality of the FO/RO was as follows: 0.3 meq. peroxides / kg oil, 230  
107 mg  $\alpha$ -tocopherol / kg, 31 mg  $\beta$ -tocopherol / kg, 151 mg  $\gamma$ -tocopherol / kg and 6 mg  $\delta$ -tocopherol /  
108 kg. The fatty acid composition of the FO/RO was as follows: 14:0, 1.7%; 16:0, 7.1%; 16:1 (n-7),  
109 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  
110 (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  
111 total content of n-3 and n-6 PUFA in the FO/RO were 17.8% and 10.9%, respectively.

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

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

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

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4 121 (Steinheim, Germany). Synperonic was purchased from CRODA (East Yorkshire, UK). All  
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6 122 solvents used were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland). The external  
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8 123 standards used for quantification of secondary oxidation products were from Sigma Aldrich  
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10 124 (Steinheim, Germany).

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### 14 15 126 **Experimental design**

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18 127 For specific details on emulsifier, oil and antioxidants applied refer to Table 1. Experiment 1 was  
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20 128 designed to evaluate the effect caffeic acid and different alkyl caffeates as antioxidant in Citrem  
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22 129 stabilized emulsions in the presence of endogenous tocopherols due to the natural content of  
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24 130 tocopherols in oil. In this experiment fish oil was used, and a storage period of 15 days selected  
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26 131 based on previous storage experiment with fish oil.

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30 132 Experiment 2 was designed to evaluate the influence of endogenous tocopherols on the efficacy of  
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32 133 caffeic acid and caffeates in Tween stabilized emulsions. Tween was selected as emulsifier, since  
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34 134 Citrem contains tocopherols. Emulsions were prepared with and without endogenous tocopherols.  
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36 135 Thus, the oil without endogenous tocopherols was stripped (removal of tocopherols). Stripping of  
37  
38 136 fish oil will increase oxidation rates tremendously. To slow down oxidation rate, a mixture of fish  
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40 137 and rapeseed oil was therefore used instead in this experiment. The storage time was selected based  
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42 138 on preliminary laboratory trials (data not shown).

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46 139 Based on experiment 1 and 2 with endogenous tocopherols, the impact of emulsifier type is  
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48 140 evaluated despite different oil type and length of storage time.

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### 52 53 142 **Removal of tocopherols from oils**

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4 143 FO/RO was stripped from tocopherols using an alumina packed glass column using hexane. For  
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6 144 further details refer to Sørensen et al [8]. The stripped oil was bottled, flushed with nitrogen and  
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8 145 stored at -80°C until use for production of emulsions. Furthermore, the absence of tocopherols in  
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10 146 the oil was checked by HPLC according to the AOCS method [13]. After oil stripping (removal of  
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12 147 tocopherols) the PV was 0.5 meq. peroxides / kg oil and tocopherols were not detected.  
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### 17 149 **Production of O/W emulsions**

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20 150 Both in experiment 1 and 2, the emulsion compositions were 5% oil, 1% emulsifier and 94% 10  
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22 151 mM sodium acetate – imidazole buffer (pH 7). Antioxidants were diluted in methanol and added in  
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24 152 concentrations of 100 µM. For the control emulsions (without antioxidant added), methanol was  
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26 153 added in same amount as used for the methanolic antioxidant solutions added to the other  
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28 154 emulsions. The short to medium chain phenolipids (C0 - C12) were added to the buffer (Citrem  
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30 155 emulsions) and buffer-emulsifier mixture (Tween emulsions), whereas the long chain phenolipids  
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32 156 (C16 – C20) were added to the oil-emulsifier mixture (Citrem emulsions) and oil (Tween  
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34 157 emulsions) before the pre-homogenisation step.  
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38 158 Preparation of emulsions for storage experiment 1 were produced with pre-emulsification (2 min,  
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40 159 Ultra-Turrax, Janke & Kunkel IKA-Labortechnik, Staufen, Germany) followed by homogenization  
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42 160 on a two-valve table homogenizer at a pressure of 800 bar (GEA Niro Soavi Spa, Parma, Italy). For  
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44 161 further details refer to Sørensen et al. [14]. Production of emulsions for storage experiment 2 were  
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46 162 pre-emulsified as in experiment 1, but homogenized on a microfluidizer (9K, Microfluidics,  
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48 163 Newton, MA, USA). Changes in the production between experiment 1 and 2 (homogenizer vs.  
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50 164 microfluidizer) were done in order to operate with smaller emulsion volumes and thereby reduce the  
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52 165 amount of phenolipids. Moreover, it became possible to cool the emulsion during production which  
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4 166 was an advantage due to the fact that the oil was stripped from tocopherols and would therefore be  
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6 167 highly susceptible to oxidation during homogenization.  
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9 168 After production emulsions (100 g) were stored in 100 mL blue cap bottles at  $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . Samples,  
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11 169 one bottle pr. code, were taken at specific time points and divided into brown glass bottles, flushed  
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13 170 with nitrogen and stored at  $-40^{\circ}\text{C}$  until analyses, except for samples used to determine droplet size,  
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15 171 these samples were measured at the sampling day without pre-freezing.  
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### 18 172 **Droplet size measurements**

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21 173 Droplet size of the oil droplets in the O/W emulsion was determined by laser diffraction  
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23 174 (Mastersizer2000, Malvern Instruments Ltd., Worcestershire, UK). Few droplets of the different  
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25 175 emulsions were suspended directly in recirculating water (2800 rpm, obscuration 12-14%). Water  
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27 176 ( $\text{RI}_{\text{water}} = 1.330$ ) and sunflower oil ( $\text{RI}_{\text{oil}} = 1.469$ ) were used in this measurements as dispersant and  
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29 177 particle, respectively. Each sample was measured in triplicate ( $n=3$ ) and results are reported as  
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31 178 surface mean diameter,  $D_{3,2}$  [15].  
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### 38 180 **Lipid extractions from O/W emulsions**

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41 181 Peroxide value (PV) and tocopherol analyses are performed on lipid extracts. Thus, the lipids were  
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43 182 extracted from the emulsions prior to these analyses according to the method described by Bligh  
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45 183 and Dyer [16] using a reduced amount of solvent [17]. For each sample code two lipid extractions  
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47 184 were performed ( $n=2$ ).  
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### 53 186 **Tocopherols**

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4 187 Lipid extracts were evaporated under nitrogen, re-dissolved in heptane and analyzed by HPLC  
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6 188 (Agilent 1100 Series, Agilent Technology, Palo Alto, CA, USA) according to the AOCS Official  
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8 189 Method Ce 8-89 [13]. A silica column (Waters (Dublin, Ireland), 150mm, 4.6mm, 3 $\mu$ m silica film)  
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10 190 was used for separation of the tocopherol homologues. This analysis was performed in duplicate on  
11  
12 191 each lipid extract and results reported as  $\mu$ g tocopherol / g emulsion.  
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### 18 193 **Primary oxidation products: Peroxide value (PV)**

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21 194 PVs in the lipid extracts were determined by colorimetric method based on formation of an iron-  
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23 195 thiocyanate complex. The colored complex was measured on a spectrophotometer at 500 nm  
24  
25 196 (Shimadzu UV1800, Shimadzu Scientific Instruments, Columbia, MD, USA) [18]. The analysis  
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27 197 was performed in duplicate and reported as meq peroxides / kg oil.  
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### 33 199 **Secondary oxidation products: Volatiles**

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36 200 Volatiles were released from the O/W emulsion using dynamic headspace (45°C for 30 min,  
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38 201 nitrogen flow of 150 mL/min). Volatiles were then collected and trapped on Tenax GR packed  
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40 202 tubes. To avoid foam and thus water on the tubes, 4 mL of antifoam (Synperonic, conc. 8 g /L  
41  
42 203 water) was added to each sample prior to the collection. Trapped volatiles were desorbed using an  
43  
44 204 automatic thermal desorber (ATD-400, Perkin Elmer, Waltham, MA, USA). The transfer line of  
45  
46 205 the ATD was connected to a gas chromatograph (Agilent 5890, Palo Alto, CA, USA) with a mass  
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48 206 selective detector (HP 5972). Volatiles were separated on a DB1701 column (30m x ID 0.25mm x  
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50 207 1 $\mu$ m film thickness, J&W Scientific, Folsom, CA, USA). The initial temperature of the oven was  
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52 208 45°C, which was kept for 5 minutes and then gradually increased as follows: 45-55°C 1.5°C/min,  
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4 209 55-90°C 2.5°C/min, 90-220°C 12°C/min and kept at 220°C for 4 minutes. Calibration curves  
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6 210 prepared from external standards was used for quantification of different volatiles. In experiment 1  
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8 211 (Table 1), different concentrations of external standard solutions were prepared and 1  $\mu$ L was  
9  
10 212 placed in Tenax tubes and analyzed. In experiment 2 (Table 1), different concentrations of external  
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12 213 standard solutions were prepared and added to fresh emulsion without antioxidant. Volatiles were  
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14 214 collected in the same way as for samples. The 2,4-heptadienal external standard appears as two  
15  
16 215 peaks in the chromatogramme, these peaks are termed A and B. Each sample code was analyzed in  
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18 216 triplicate (n=3) and results reported as ng volatile / g emulsion.  
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## 25 218 **Partitioning**

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28 219 Partitioning of caffeic acid and the different alkyl caffeates in buffer/oil, emulsifier/buffer and in  
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30 220 O/W emulsion was measured according to the method described by Schwarz et al. [19] with  
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32 221 modifications as described elsewhere. With this method it is assumed that the partitioning of the  
33  
34 222 antioxidants, equilibria reached, is not disrupted by centrifugation. For further details refer to  
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36 223 Sørensen et al. [20]. In short, the concentration of antioxidants was measured in the separated  
37  
38 224 aqueous phase of 3 different systems: buffer / oil (FO or FO/RO), buffer / emulsifier (Citrem or  
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40 225 Tween80) and 5% O/W emulsions. Antioxidants were dissolved in methanol and added in a  
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42 226 concentration of 100  $\mu$ M. Separation of the aqueous phase was carried out 24 h after production of  
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44 227 the different systems.  
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## 51 229 **Determination of concentration of added antioxidant**

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4 230 Caffeic acid and alkyl caffeates in the aqueous phases were analyzed by HPLC (Agilent 1100  
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6 231 Series, Agilent Technology, CA, USA) with a C18 Thermo Hypersil® ODS (250x4.6 mm, 5 $\mu$ )  
7  
8 232 column and using a gradient elution at a flow rate of 1 mL/min. Solvent A was 3 mM phosphoric  
9  
10 233 acid and solvent B was methanol. Gradient condition: 0-30 min 0-100% B, 30-40 min 100% B and  
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12 234 40-45 min 100-0% B. Injection volume was 20  $\mu$ L. Caffeic acid and different alkyl caffeates were  
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14 235 quantified by calibration curves of these compounds dissolved in methanol.  
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### 237 **Conjugated autoxidizable triene (CAT) Assay**

238 Stock solutions of the different compounds: caffeic acid, caffeates and trolox were prepared in  
239 methanol. The CAT assay was performed as described in Sørensen et al. [8] with a modification.  
240 This assay was performed with non-stripped tung oil, hence, the assay contained tocopherol and the  
241 evaluated antioxidant in a mixture. Each microplate well contained a microemulsion with the  
242 following composition: 115  $\mu$ M tung oil, 17  $\mu$ M Brij 35, 1 mM AAPH and antioxidant (caffeic  
243 acid, caffeates or Trolox) in various concentrations. The progress of lipid oxidation was followed by  
244 measuring the decrease in absorbance at 273 nm. Each antioxidant concentration was measured in  
245 triplicate on the plate and via independent measurements (two different microplates), n = 6. Results  
246 were expressed as CAT value (mean  $\pm$  SD). This method was developed by Laguerre et al. [21]. For  
247 further details about the calculations refer to Laguerre et al. [4, 21].  
248

### 249 **Data treatment**

250 **Statistics.** The results obtained were analyzed using one- and two-way ANOVA (GraphPad Prism,  
251 Version 4.01, GraphPad Software Inc). Bonferroni multiple comparison post-test was used to  
252 determine significant differences between samples or storage times. The significance level applied

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4 253 was 95% ( $p < 0.05$ ). Significant difference between samples is denoted with different superscripts  
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6 254 (i.e. a, b, c ect.).

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9 255 ***Inhibition percentages.*** Since different oil and emulsifiers can influence amount of lipid oxidation,  
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11 256 inhibition percentages were used to compare the efficacy of the antioxidants in the different  
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13 257 emulsion systems. The antioxidants inhibition percentages were calculated according to the  
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15 258 following equation:

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$$\text{Inhibition [\%]} = \left( \frac{\text{Emulsion}_{\text{Control}} - \text{Emulsion}_{\text{Antioxidant}}}{\text{Emulsion}_{\text{Control}}} \right) \times 100$$

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21  
22 260  $\text{Emulsion}_{\text{Control}}$  and  $\text{Emulsion}_{\text{Antioxidant}}$  are emulsions without antioxidant and emulsion with  
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24 261 antioxidant added, respectively. The antioxidant has a prooxidative effect if the inhibition  $< -5\%$   
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26 262 and an antioxidative effect  $> 5\%$ . The antioxidative effect can be categorized as weak (5-20%),  
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28 263 intermediate (20-50%) or strong ( $> 50\%$ ) effects [22].

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34 265 ***Multivariate data analysis, Principal Component Analysis (PCA).*** Inhibition percentages  
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36 266 calculated from results obtained from PV, volatiles and tocopherol analysis were subjected to PCA  
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38 267 using Unscrambler version X10.3 (Camo, Oslo, Norway). The PCA model was built on inhibition  
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40 268 percentages calculated on the average of the measured data. Full cross validation was used to  
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42 269 validate the model. A PCA allows detection of similarities and dissimilarities between the different  
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44 270 samples in a score plot, whereas correlations between the measured variables are visualized in a  
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46 271 loadings plot. Connecting these plots in this case shows the degree of oxidation between the  
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48 272 different samples.



## 273 **Results**

### 274 **Physical stability of the emulsions**

275 The physical stability of the emulsions was evaluated by following the oil droplet size (D<sub>3,2</sub>,  
276 (surface weighted mean)) over time. With Citrem as emulsifier (Exp. 1), the droplet size was  
277 between 101 – 114 nm for the different Citrem stabilized emulsions (data not shown). The emulsion  
278 prepared with Tween as emulsifier (Exp. 2), non-stripped FO/RO and stripped FO/RO had droplet  
279 sizes between 124 – 131 nm and 126 - 131 nm, respectively (data not shown). Emulsions produced  
280 on the homogenizer (Exp. 1), resulted in slightly smaller oil droplets than emulsions produced on  
281 the microfluidizer (Exp. 2). The small differences in oil droplet sizes, however significant, for  
282 Citrem and Tween stabilized emulsions is suggested to be due to the different emulsification  
283 equipment applied, the different emulsifiers used or a combination. Changes in droplet size during  
284 storage were minor, but significant. The changes ranged from -6 nm to +2 nm in size. Since, the oil  
285 droplets mainly had a slight decrease in size during storage, the changes is suggested to be due to  
286 day to day instrumental variation. Hence, all emulsions were physically stable during the whole  
287 storage time, 42 hours, 6 and 15 days, respectively.

288

### 289 **Partitioning of caffeic acid and caffeates**

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

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4 296 octyl- (C8) and dodecyl caffeates (C12) were detected in the aqueous phase and the concentrations  
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6 297 were not significantly different from that of butyl caffeate (C4). The concentration of caffeic acid  
7  
8 298 and methyl caffeate in the aqueous phase was significantly lower when Tween was applied as  
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10 299 emulsifier than when Citrem was applied. Moreover, the concentration of butyl caffeate tended to  
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12 300 be lower (not significant) in the aqueous phase with Tween as emulsifier than with Citrem as also  
13  
14 301 observed for caffeic acid and methyl caffeate. These partitioning results indicated interactions  
15  
16 302 between antioxidants and emulsifier to a higher extent with Tween than Citrem, thus, resulting in a  
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18 303 higher concentration of antioxidant at the interface with Tween than Citrem as emulsifier. In  
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20 304 addition, the results indicated that there was a tendency to a lower concentration of caffeic acid and  
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22 305 methyl caffeate in the aqueous phase of the non-stripped Tween80 emulsion, i.e. when endogenous  
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24 306 tocopherol was present. However, the differences were not significant (Table 2).  
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### 31 308 **Efficacy of caffeic acid and caffeates in emulsions**

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34 309 The antioxidant efficacy of caffeic acid and caffeates were evaluated in the three different  
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36 310 emulsions shown in Table 1 and in a modified CAT assay (modification: non-stripped tung oil  
37  
38 311 applied) to investigate the effect of the presence of endogenous tocopherols in this assay. The  
39  
40 312 efficacy of caffeic acid and caffeates was evaluated from the measured lipid oxidation during  
41  
42 313 storage. Lipid oxidation was followed by measuring PV (primary oxidation product) and volatiles  
43  
44 314 (secondary oxidation products). Volatile oxidation products that increased during storage were  
45  
46 315 identified and quantified. The quantified volatiles were markers of lipid oxidation products, most of  
47  
48 316 them originated from oxidation of n-3 PUFAs.  
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51  
52 317 *Citrem stabilized emulsions with endogenous tocopherols present.* The PV in this experiment  
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54 318 increased to 20-35 meq. peroxides / kg oil depending on the antioxidant treatment during the 15  
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4 319 days of storage (data not shown). The concentration of volatiles after 15 days of storage  
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6 320 depended upon the specific volatile quantified and the antioxidant applied (1-penten-3-one 20-  
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8 321 60 ng / g emulsion, 1-penten-3-ol 100-300 ng / g emulsion, hexanal 70-150 ng / g emulsion, 4-  
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10 322 heptenal 10-25 ng / g emulsion, 2,4-heptadienal 4000-6000 ng / g emulsion and nonanal 30-70 ng /  
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12 323 g emulsion).

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15 324 A PCA of the PVs,  $\alpha$ -tocopherol and volatile compounds measured during storage explained 71%  
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17 325 of the variation in the obtained results by the first two principal components (PCs), Figure 1. The 3  
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19 326 other tocopherols ( $\beta$ -,  $\delta$ -, and  $\gamma$ -tocopherols) were excluded from the PCA model due to no or minor  
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21 327 changes during storage in the different emulsions. Figure 1A shows the correlation loadings i.e.  
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23 328 graphical mapping of the measured variables. Generally, all the volatiles were located in quadrant 1  
24  
25 329 (top-right part) and 4 (bottom-right part), PVs in quadrant 2 (top-left part) closer to PC 2 and  $\alpha$ -  
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27 330 tocopherols in quadrant 2 and 3 (bottom-left part) a long PC 1. Thus, the first PC clearly described  
28  
29 331 lipid oxidation with tocopherol in the left side and volatiles in the right side. Comparing Figure 1A  
30  
31 332 with 1B, the scores, reveals the differences in efficacy between caffeic acid and caffeates. Butyl  
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33 333 caffeate was prooxidative due to its location in the 4th quadrant i.e. high concentration of many of  
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35 334 the volatile compounds. Octyl caffeate also acted as a prooxidant, however, not as strong as butyl  
36  
37 335 caffeate. Furthermore, hexadecyl caffeate's location in first quadrant, but close to PC 2, indicates a  
38  
39 336 slight prooxidative effect. Dodecyl and eicosyl caffeates seemed to have no effect due to their  
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41 337 proximity to the control emulsion in the 2nd quadrant. The location of caffeic acid in the 3 quadrant  
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43 338 of the scores plot away from the volatile compounds (1 and 4 quadrant) indicates that this  
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45 339 antioxidant was the most efficient followed by methyl caffeate. However, caffeic acid and methyl  
46  
47 340 caffeate were not strong antioxidants, since some of the measured volatiles were present in higher  
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49 341 concentration in these emulsions than in the control emulsion (Table 3, Inhibition percentages, Raw  
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51 342 data not shown).

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4 343 ***Tween stabilized emulsions with endogenous tocopherols present.*** In the Tween stabilized  
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6 344 emulsion with endogenous tocopherols present, the PV increased to 5-30 meq. peroxides / kg oil  
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8 345 depending on the antioxidant treatment during the 6 days of storage. The concentration of volatiles  
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10 346 after 6 days of storage dependent upon the specific volatile quantified and the antioxidant applied  
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12 347 (1-penten-3-one 2-10 ng / g emulsion, 1-penten-3-ol 5-30 ng / g emulsion and 2,4-heptadienal 50-  
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14 348 350 ng / g emulsion).

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17 349 A PCA of the PVs,  $\alpha$ -tocopherol and volatile compounds (1-penten-3-one, 1-penten-3-ol and 2,4-  
18  
19 350 heptadienal) measured during storage explained 82% of the variation in the obtained results by the  
20  
21 351 first two PCs (Figure 2). Similar to the other PCA model on Citrem stabilized emulsions, the 3 other  
22  
23 352 tocopherol homologues ( $\beta$ -,  $\delta$ -, and  $\gamma$ -tocopherols) were excluded from the PCA model due to no or  
24  
25 353 minor changes during storage in the different emulsions. The PV and all the volatiles were located  
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27 354 to the left in the plot of the correlation loadings (Figure 2A). Tocopherols were located opposite to  
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29 355 the PV and volatiles. From the correlation loadings plot it is clear that the first PC describe lipid  
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31 356 oxidation with tocopherol in the right side and volatiles in the left side, thus, increased lipid  
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33 357 oxidation moving from the right to the left in the plot. Scores plot (Figure 2B) reveals differences in  
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35 358 efficacy between caffeic acid and caffeates in the tween stabilized emulsions. The control emulsion  
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37 359 was located to the left side of the PC 2 axis in the scores plot and all emulsions with antioxidant  
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39 360 added were located to the right of the PC 2 axis except emulsion with butyl caffeate added (Figure  
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41 361 2B). In connection with correlation loadings plot, this indicates that all the emulsions with  
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43 362 antioxidant added acted as antioxidant in the tween stabilized emulsions. As described above, butyl  
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45 363 caffeate was located opposite to the PC 2 axis compared to the other emulsions with antioxidant  
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47 364 added, this is explained by the higher amount of 2,4-heptadienal at day 6 in this emulsion (Figure  
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49 365 2). Butyl caffeate worked as antioxidant for all other oxidation parameters measured; however, it  
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51 366 was the least efficient due to higher amount of the measured oxidation parameters compared to the  
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4 367 other antioxidant applied (Table 3). Moreover, the PCA model reveals that caffeic acid without  
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6 368 esterification was more efficient in tween stabilized emulsions followed by methyl caffeate (short  
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8 369 chain esterification) due to their location most far away from the oxidation parameters measured.  
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10 370 Raw data supported the PCA model.

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13 371 ***Tween80 stabilized emulsions without endogenous tocopherols present.*** In the Tween stabilized  
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15 372 emulsion with endogenous tocopherols present, the PV increased from 8 to 14 meq. peroxides / kg  
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17 373 oil without antioxidant added during the 42 hours of storage; whereas, the PV increased from 2 to 8  
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19 374 and 1 to 4 meq. peroxides / kg oil with caffeic acid and eicosyl caffeates, respectively. The other  
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21 375 antioxidant treatments resulted in no increase in PV during storage. The concentration of volatiles  
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23 376 after 42 hours of storage dependent upon the specific volatile quantified and the antioxidant applied  
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25 377 (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  
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27 378 / g emulsion), which was much lower than in the Citrem stabilized emulsions.

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31 379 A PCA of the PVs and volatile compounds (1-penten-3-one, 1-penten-3-ol and 2,4-heptadienal)  
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33 380 measured during storage explained 83% of the variation in the obtained results by the first to  
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35 381 principal components (PCs), Figure 3. All the volatiles were located in quadrant 1 (top-right part)  
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37 382 and 4 (bottom-right part). The first PC clearly describes lipid oxidation (right side) versus no lipid  
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39 383 oxidation (left side), whereas, PC 2 describes the development of lipid oxidation over time, with PV  
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41 384 and volatiles in the beginning of the storage period located in the top of the plot and in the bottom  
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43 385 of the plot after 42 days (Figure 3A). Comparing Figure 3A with 3B, the scores plot, it is observed  
44  
45 386 that control emulsion and emulsion with caffeic acid is located in the same side as the oxidation  
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47 387 parameters measured. Thus, caffeic acid is acting as a prooxidant in Tween stabilized emulsions  
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49 388 without endogenous tocopherol present. Esterification of caffeic acid in Tween stabilized emulsion  
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51 389 without tocopherols improved its antioxidative properties, since all caffeates evaluated were acting  
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53 390 as antioxidants. However, it seems like caffeic acid esterified with C20 was slightly less efficient  
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4 391 than the other esters evaluated (Table 3). Raw data supports the observation from the PCA model  
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6 392 (data not shown).  
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9 393 ***Comparison of the influence of emulsifier type - Citrem versus Tween80 stabilized emulsions.***

10  
11 394 Table 3 shows calculated inhibition percentages for caffeic acid and caffeates. It is clear that the  
12  
13 395 emulsifier impacted the efficacy of the antioxidants added. In Citrem stabilized emulsions, caffeic  
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15 396 acid and methyl caffeate were the only ones acting as antioxidants. Their antioxidative effect in this  
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17 397 model emulsion was weak to intermediate and they even promoted the formation of certain  
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19 398 volatiles. In contrast, caffeic acid and all the evaluated caffeates in Tween stabilized emulsions  
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21 399 acted as antioxidants. Caffeic acid was the strongest antioxidant followed by methyl caffeate.  
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25 400 ***Comparison of the influence of the presence of endogenous tocopherols.*** Calculated inhibition  
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27 401 percentages for selected oxidation variables measured in Tween stabilized emulsions with and  
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29 402 without endogenous tocopherols are presented in Table 3. A clear difference in the efficacy of  
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31 403 antioxidants was that caffeic acid acted as a strong antioxidant when tocopherols were present,  
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33 404 whereas, it acted as intermediate to strong prooxidant without tocopherols in this model emulsion.  
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35 405 In addition, the caffeates acted as stronger antioxidants without tocopherols compared to the same  
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37 406 model emulsion with tocopherols present (Table 3). Caffeates with chain lengths between C1 and  
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39 407 C18 were all strong antioxidants, whereas the antioxidant efficacy was decreased when the chain  
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41 408 length was increased to C20.  
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45 409 ***Antioxidant efficacy in a modified CAT assay.*** The CAT assay is an assay developed to measure  
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47 410 the efficacy of antioxidants in a micro emulsion system without endogenous tocopherols present.  
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49 411 The assay was slightly modified to investigate the efficacy of caffeic acid and caffeates in the  
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51 412 presence of endogenous tocopherols. The results are shown in Figure 4 together with results  
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53 413 obtained earlier with an unmodified CAT assay [8]. When tocopherol was present the antioxidative  
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4 414 efficacies of caffeic acid, methyl-, butyl-, octyl- and dodecyl caffeates were not significantly  
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6 415 different. This finding is different from earlier results obtained without the presence of tocopherols,  
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8 416 where octyl- and dodecyl caffeates exerted a significantly higher efficacy than caffeic acid and the  
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10 417 other caffeates evaluated. Furthermore, the efficacy of octyl- and dodecyl caffeates without  
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12 418 tocopherols present was also significantly higher than when tocopherols were present.  
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For Peer Review

## 419 **Discussion**

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

426 *Impact of emulsifier on the efficacy of caffeic acid and caffeates.* In the present study, caffeic acid  
427 and caffeates were more efficient antioxidants in Tween stabilized emulsions than in Citrem  
428 stabilized emulsions. This partly supports earlier findings with the same emulsifiers and caffeic acid  
429 in 10% O/W emulsions, where caffeic acid in Citrem stabilized emulsions promoted the formation  
430 of volatiles and no effect on lipid oxidation or slightly antioxidative effect of caffeic acid was  
431 observed in Tween stabilized emulsions [23]. Besides different oil concentration in the previous and  
432 current studies, antioxidant concentration (5.5 fold lower in this study) and oil / emulsifier ratio  
433 were also different, which can have an influence on the differences observed in these studies.  
434 Independently of emulsifier type, caffeic acid was performing better than the caffeates. Contrary,  
435 the effect of the caffeates was affected by the emulsifier applied. To our knowledge only few  
436 studies related to the antioxidative effect of caffeic acid and caffeates in emulsions have been  
437 published [9, 24, 25]. These studies did not compare the effect of the emulsifier type applied.  
438 However, one of them compared the effect of caffeates in mayonnaise and milk, where not only the  
439 emulsifier type is different but the entire emulsion system. The efficiency of the caffeates was  
440 affected by the type of emulsion system [9]. Results obtained in the present study also demonstrated  
441 that changing the emulsifier affected the antioxidative effect and rank order of caffeates.  
442 Experiments performed with gallic acid and ethyl gallate have also shown that changing emulsifier



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4 443 affects the partitioning of the antioxidants and the resulting antioxidant activity measured in  
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6 444 emulsion systems [26]. The different emulsifier evaluated was SDS, CTAB, Brij58 and PHLC, and  
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8 445 their partitioning study revealed increased solubility effect of the emulsifiers in the following order:  
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10 446 PHLC < SDS < Brij58 < CTAB. The antioxidant activity of gallic acid and ethyl gallate based on  
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12 447 the formation of hydroperoxides and hexanal increased in the following order: CTAB (no activity  
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14 448 measured) < Brij58 < PHLC < SDS. Gallic acid only showed antioxidant activity with PHLC  
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16 449 stabilized emulsions. This was a reverse order compared to the partitioning measured. Hence, it was  
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18 450 suggested that the increased partitioning into the emulsifier layer and lipid counteract the hydrogen-  
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20 451 donating ability, and lower the activity of the antioxidants [26]. Moreover, Pekkarinen et al. [27]  
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22 452 evaluated antioxidative effect and partitioning of phenolics in different systems. Interaction between  
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24 453 caffeic acid and Tween 20 differed from other phenolics such as vanillic acid, ferulic acid and  
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26 454 sinapic acid evaluated, since Tween 20 exhibited higher solubilisation capacity for caffeic acid than  
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28 455 for other phenolic acids. Additionally, Pekkarinen et al. [27] concluded that these antioxidant-  
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30 456 emulsifier interactions have a strong influence on the partitioning of antioxidants. The partitioning  
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32 457 result obtained in this study confirmed that Citrem and Tween as emulsifiers results in differences  
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34 458 in the partitioning of caffeic acid and caffeates. Less caffeic acid and caffeates were present in the  
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36 459 aqueous phase when Tween was applied. This clearly demonstrated stronger antioxidant-emulsifier  
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38 460 interaction with Tween compared to Citrem. Moreover, Citrem is an anionic emulsifier, thus, the  
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40 461 interface is negatively charged and will repel negatively charged antioxidants i.e. caffeic acid,  
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42 462 which could explain why caffeic acid interacted less with Citrem than with Tween. An explanation  
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44 463 for the stronger interactions with Tween may be the molecular structure of the emulsifiers, since  
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46 464 Tween is a larger and more bulky molecule than Citrem; however, this has to be further evaluated.  
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48 465 Furthermore, Schwarz et al. [19, 28, 29] evaluated partitioning of different antioxidants in dispersed  
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50 466 lipid systems with different emulsifiers. Significant differences were observed in partitioning of the  
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4 467 antioxidants between phases, both as a function of pH and emulsifier type and concentration. It was  
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6 468 concluded from the results that determination of antioxidant partitioning may be an important tool  
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8 469 to select antioxidants structurally designed to localize at the surfaces [19], however, the partitioning  
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10 470 of the antioxidants cannot alone explain the measured antioxidant activity in emulsions [29].  
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12 471 Results from another study by Schwarz et al. [30] evaluating antioxidant activity of antioxidants  
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14 472 with different lipophilicity in bulk oil, O/W and W/O emulsions with different emulsifiers led to the  
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16 473 assumption that differences in antioxidant activity for the same emulsion type might be additionally  
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18 474 influenced by interaction with the emulsifier dominating the interfaces in the emulsion system [30].  
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21 475 The obtained results for the two emulsifiers together with the partitioning study may also here lead  
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23 476 to the assumption that emulsifier-antioxidant interactions (e.g. hydrogen bonding) affected the  
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25 477 antioxidant activity of the caffeates. However, the type of interactions and the impact of  
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27 478 antioxidant-emulsifier interactions on the radical scavenging activity have to be studied in more  
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29 479 details to make further conclusions.

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33 480 ***Impact of endogenous tocopherols on the efficacy of caffeic acid and caffeates.*** The presence of  
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35 481 endogenous tocopherol not only changed the antioxidant activity of caffeic acid and caffeates in  
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37 482 both the storage experiment and in the CAT assay, but also their partitioning in the emulsion  
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39 483 system. A tendency to less caffeic acid and caffeates (C1, C4 and C12) present in the aqueous phase  
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41 484 with endogenous tocopherol in the emulsion system was observed. This may indicate some  
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43 485 interactions between tocopherol and caffeic acid / caffeates both for the antioxidative effect and  
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45 486 localization in the emulsion system. The use of a combination of antioxidants to produce synergistic  
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47 487 interaction has been reported earlier e.g. tocopherol regeneration by ascorbic acid, polyphenols and  
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49 488 flavonoids [31-34]. Panya et al. [34] carried out the only study investigating interactions between  
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51 489 tocopherol and a phenol (rosmarinic acid) and its alkyl esters (rosmarinates, C4, C12 and C20) in  
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53 490 Tween20 stabilized emulsions. Rosmarinic acid exhibited strongest synergistic interaction with  
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4 491 tocopherol, and C4 and C12 esters exhibited small synergistic interaction. An antagonistic  
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6 492 interaction was observed with C20 ester and tocopherol. Thus, the more hydrophilic rosmarinic acid  
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8 493 exhibited more interactions with the tocopheryl radical than the esters. In the present study, the  
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10 494 emulsion with the more hydrophilic caffeic acid exhibited better oxidative stability than the  
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12 495 emulsions with the esters (more hydrophobic antioxidants) when tocopherol was present as also  
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14 496 observed with rosmarinic acid and rosmarinates. Actually, caffeic acid turned from being  
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16 497 prooxidative without tocopherol present to being the most efficient antioxidant with endogenous  
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18 498 tocopherol present in Tween stabilized emulsion. In emulsions, the majority of the emulsifier is  
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20 499 accumulated at the oil-water interface. However, a part of the emulsifier is not associated with the  
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22 500 oil-water interface if the emulsifier concentration is above the CMC (critical micellar concentration,  
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24 501 CMC Tween80 13-15 mg/L, Sigma) and will form micelles in the aqueous phase. In this study, the  
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26 502 concentration of Tween was much higher than CMC (10 g/L). The decreased antioxidant efficiency  
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28 503 of the different caffeates compared with caffeic acid is suggested to be due to the solubilisation of  
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30 504 caffeates in Tween micelles, thus, localized away from the interface unable to inhibit lipid  
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32 505 oxidation. Although, micelles are not isolated structures, they compromise structures that are in  
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34 506 dynamic equilibrium with other structures in the emulsion system. This means that components can  
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36 507 be exchanged between the different structures i.e. between micelles and emulsion droplets [35, 36].  
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38 508 Thus, more studies are needed to further elucidate the differences observed in partitioning and  
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40 509 antioxidant effect in the presence of endogenous tocopherols.  
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45 510 Additionally, it is assumed that caffeic acid is located in close proximity to the interface where it  
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47 511 regenerates tocopherol at the interface in spite of the repelling effect of two negatively charged  
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49 512 compounds (Citrem and caffeic acid). The proposed partial location of tocopherol at the interface is  
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51 513 supported by Jacobsen et al. [37] who reported that ca. 6 % of the alpha-tocopherol present in  
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53 514 mayonnaise was located at the interface whereas the remaining tocopherol was located in the oil  
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4 515 phase. Therefore, lipophilization is not needed to improve the oxidative stability of these emulsions,  
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6 516 due to tocopherols location at the interface. *Antioxidant hypotheses and efficacies of caffeic acid*  
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8 517 *and caffeates*. The polar paradox was not confirmed in this study since caffeic acid was more  
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10 518 efficient as antioxidant than caffeates in Citrem stabilized emulsions when endogenous tocopherol  
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12 519 was present. In Tween stabilized emulsions with endogenous tocopherol caffeic acid followed by  
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14 520 methyl caffeate were the most efficient antioxidants, whereas, caffeic acid acted as a prooxidant  
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16 521 when tocopherol was not present. No cut-off effect was observed for the lipophilized caffeic acid,  
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18 522 since the most efficient antioxidant in both Citrem and Tween stabilized emulsions when tocopherol  
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20 523 was present was C0 (caffeic acid). A similar finding was observed for the CAT assay with  
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22 524 endogenous tocopherol present. Without endogenous tocopherol present, the caffeates were most  
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24 525 efficient antioxidants in Tween stabilized emulsions, a cut-off effect was found at C16. However,  
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26 526 the efficiency of C20 was still an intermediate to strong antioxidant. The CAT assay showed a cut-  
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28 527 off effect at around C8 and C12 with no endogenous tocopherol present. The partitioning  
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30 528 experiment clearly showed an effect of the chain length, with less antioxidant present in the aqueous  
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32 529 phase with increasing antioxidant lipophilicity. Pekkarinen et al. [27] observed that the proportion  
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34 530 of antioxidant solubilized in the lipid phase and particularly in the interface did not necessarily  
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36 531 reflect the efficiency of the antioxidant. It was assumed from their evaluation of antioxidant activity  
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38 532 and partitioning that specific interactions of the antioxidant with other compounds e.g. emulsifiers,  
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40 533 and intermolecular hydrogen bonds may play an important role in reducing antioxidant activity.  
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42 534 Furthermore, antioxidant-emulsifier interaction has a strong influence on partitioning of the  
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44 535 antioxidant. It is suggested that the caffeates interacts with the emulsifier in form of micelles when  
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46 536 endogenous tocopherols are present, resulting in reduced or no antioxidant activity. When  
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48 537 endogenous tocopherols are not present the caffeates is more likely to be solubilized at the interface  
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4 538 instead of the tocopherols. However, this has to be evaluated more in depth to conclude further on  
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6 539 these differences in partitioning and antioxidant activity with the impact of endogenous tocopherols.  
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8  
9 540 **Conclusions.** Partitioning and antioxidant activity of caffeic acid and caffeates were influenced  
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11 541 both by the emulsifier type and the presence of endogenous tocopherols. Thus, this study clearly  
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13 542 demonstrated different emulsifier-antioxidant and antioxidant-antioxidant interactions that affected  
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15 543 the efficacy of the evaluated caffeic acid and caffeates as antioxidant in emulsions. The hypotheses  
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17 544 about antioxidant in emulsions are based on simple emulsions systems without the presence of  
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19 545 tocopherols. However, the impact of the presence of tocopherols on the efficacy of other  
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21 546 antioxidants is important since most food systems contain tocopherol.  
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11 550 and Citrem, respectively.  
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15  
16 552 food systems – Effect of alkyl chain length and concentration” with project no 10-093655 financed  
17  
18 553 by the Danish Research Council, Technology and Production.  
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20  
21 554 The authors declare no conflicts of interest.  
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4 659 **Figure legends**  
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7 660 **Figure 1** PCA build on results obtained from PV, volatiles (1-penten-3-one, 1-penten-3-ol, 4-  
8 heptenal, 2,4-heptadienal, hexanal and nonanal) and  $\alpha$ -tocopherols measured on Citrem stabilized  
9 emulsions during storage (15 days) using full cross validation. A) Correlation loadings and B)  
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11 662 Scores plot. Abbreviations for sample codes refer to Table 1.  
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16 664 **Figure 2** PCA build on results obtained from PV, volatiles (1-penten-3-one, 1-penten-3-ol and 2,4-  
17 heptadienal) and  $\alpha$ -tocopherols measured on Tween80 stabilized emulsions during storage (6 days)  
18 using full cross validation. A) Correlation loadings and B) Scores plot. Abbreviations for sample  
19 665  
20 666 codes refer to Table 1.  
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25 668 **Figure 3** PCA build on results obtained from PV and volatiles (1-penten-3-one, 1-penten-3-ol and  
26 2,4-heptadienal) measured on Tween80 stabilized emulsions during storage (42 hours) using full  
27 cross validation. A) Correlation loadings and B) Scores plot. Abbreviations for sample codes refer  
28 669  
29 670 to Table 1.  
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35 672 **Figure 4** CAT Value of caffeic acid and caffeates (C1-C16) measured in the concentration range of  
36 0.5 – 2  $\mu$ M. ● CAT Values determined without endogenous tocopherols (normal condition for the  
37 673  
38 CAT assay, published in Sørensen et al. [8]) and ○ CAT Values determined with endogenous  
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40 tocopherols (modified CAT assay).  
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677 **Table 1** Experimental design of experiment 1 and 2.

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

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

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681 **Table 2** Concentration [ $\mu\text{M}$ ] of caffeic acid and caffeates (Methyl, Butyl, Octyl and Dodecyl) measured in the aqueous phase of different  
 682 systems: 95% Buffer / 5% Oil, 99% Buffer / 1% Emulsifier and Emulsion (5% Oil, 1% Emulsifier and 94% Buffer). Citrem and Tween80  
 683 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 $\pm$ 3.1 <sup>a,b,x</sup>	91.5 $\pm$ 3.6 <sup>b,x</sup>	91.5 $\pm$ 7.1 <sup>b,x</sup>	93.7 $\pm$ 11.4 <sup>b,x</sup>	75.4 $\pm$ 7.0 <sup>c,x</sup>	74.0 $\pm$ 4.3 <sup>c,x</sup>	111 $\pm$ 2.6 <sup>a,x</sup>	78.4 $\pm$ 10.1 <sup>c,x</sup>
CA C1	86.2 $\pm$ 0.9 <sup>a,y</sup>	53.3 $\pm$ 3.0 <sup>b,y</sup>	43.8 $\pm$ 8.2 <sup>b,y</sup>	82.8 $\pm$ 1.9 <sup>a,x</sup>	15.4 $\pm$ 3.3 <sup>c,y</sup>	10.1 $\pm$ 2.5 <sup>c,y</sup>	91.1 $\pm$ 2.6 <sup>a,y</sup>	15.3 $\pm$ 3.1 <sup>c,y</sup>
CA C4	11.4 $\pm$ 0.4 <sup>a,b,z</sup>	4.09 $\pm$ 0.6 <sup>a,b,c,z</sup>	3.39 $\pm$ 0.5 <sup>b,c,z</sup>	11.3 $\pm$ 0.7 <sup>a,b,y</sup>	2.10 $\pm$ 0.3 <sup>c,z</sup>	< detection	13.0 $\pm$ 1.1 <sup>a,z</sup>	< detection
CA C8	< detection	< detection	< detection	< detection	2.60 $\pm$ 3.6 <sup>z</sup>	< detection	< detection	< detection
CA C12	< detection	< detection	< detection	2.00 $\pm$ 0.6 <sup>b,z</sup>	1.10 $\pm$ 0.4 <sup>z</sup>	< detection	9.70 $\pm$ 4.2 <sup>a,z</sup>	1.10 $\pm$ 0.2 <sup>b,y</sup>

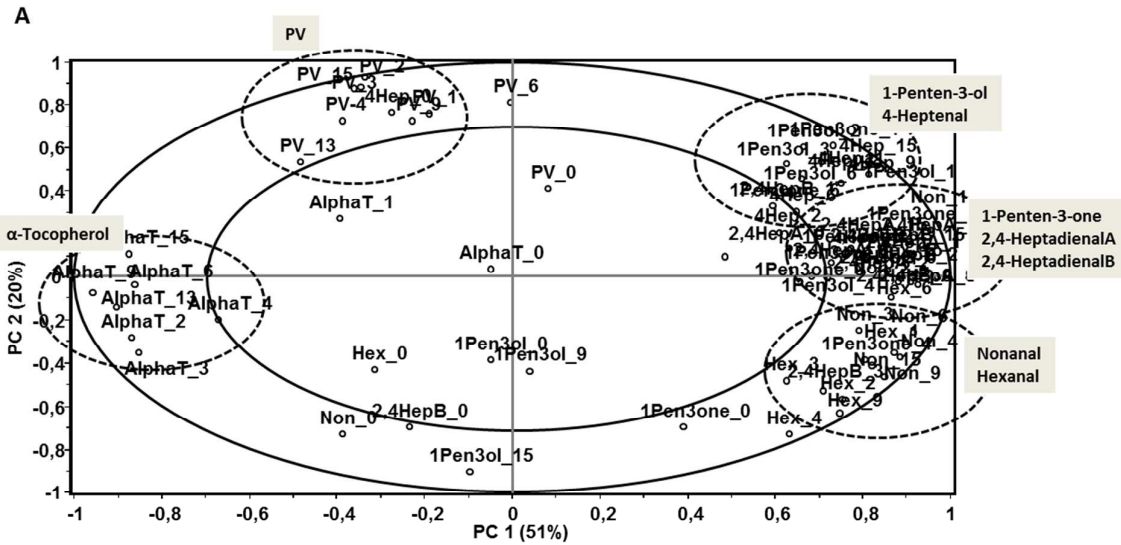
684 Different letters in superscript indicate significant differences. Significant differences within a row i.e. same antioxidant but different systems are denoted with a,b and  
 685 c, whereas significant differences within a column i.e. same system but different antioxidant are denoted with x, y and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

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

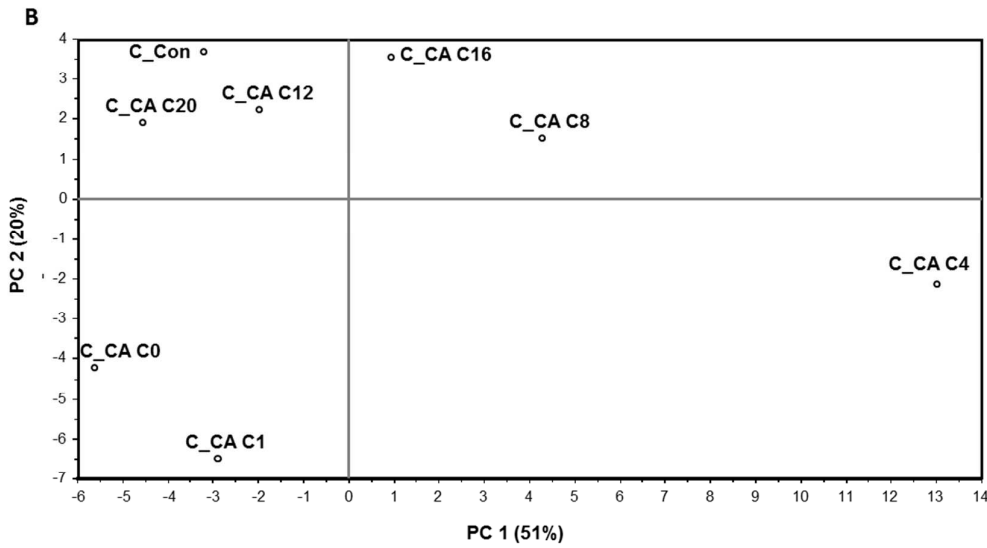
AO	Citrem (Day 6)				Tween with tocopherols				Tween without tocopherols			
	PV	1Pen3ol	1Pen3one	2,4HepA	PV	1Pen3ol	1Pen3one	2,4HepA	PV	1Pen3ol	1Pen3one	2,4HepA
CA	50	39	3	44	79	83	86	86	34	-63	-55	-48
C1	32	1	-210	-27	60	60	69	11	89	97	85	89
C4	21	-119	-502	-143	47	61	33	-10	89	99	83	93
C8	-28	-174	-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								

689 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  
 690 standard appears as two peaks in the chromatogramme, these peaks are termed A and B (here only 2,4HepA presented).

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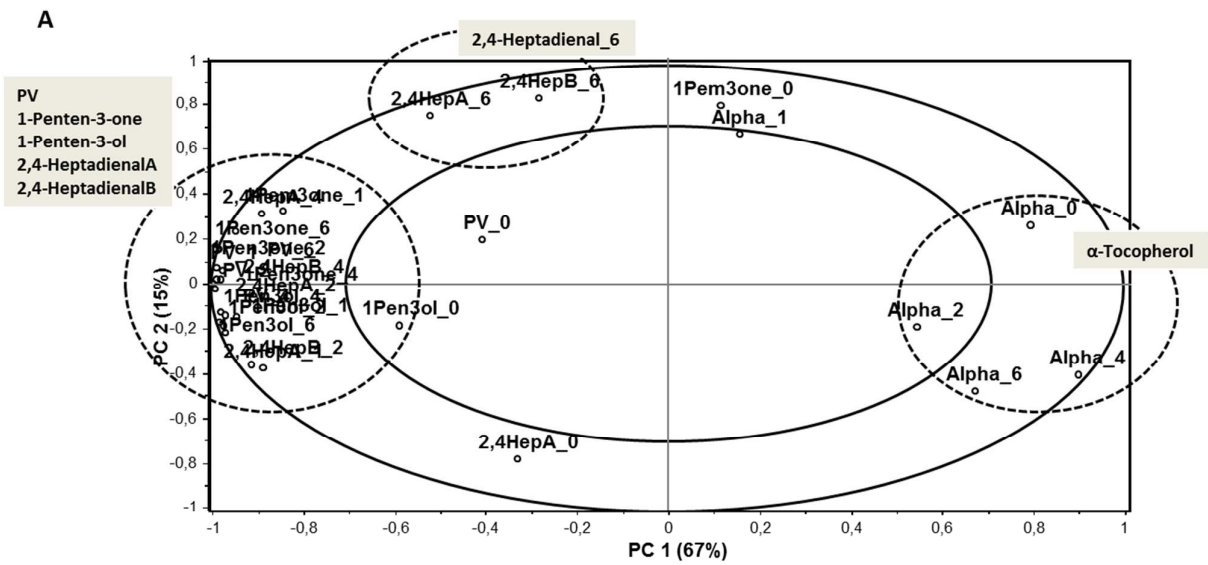


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694 **Figure 1**

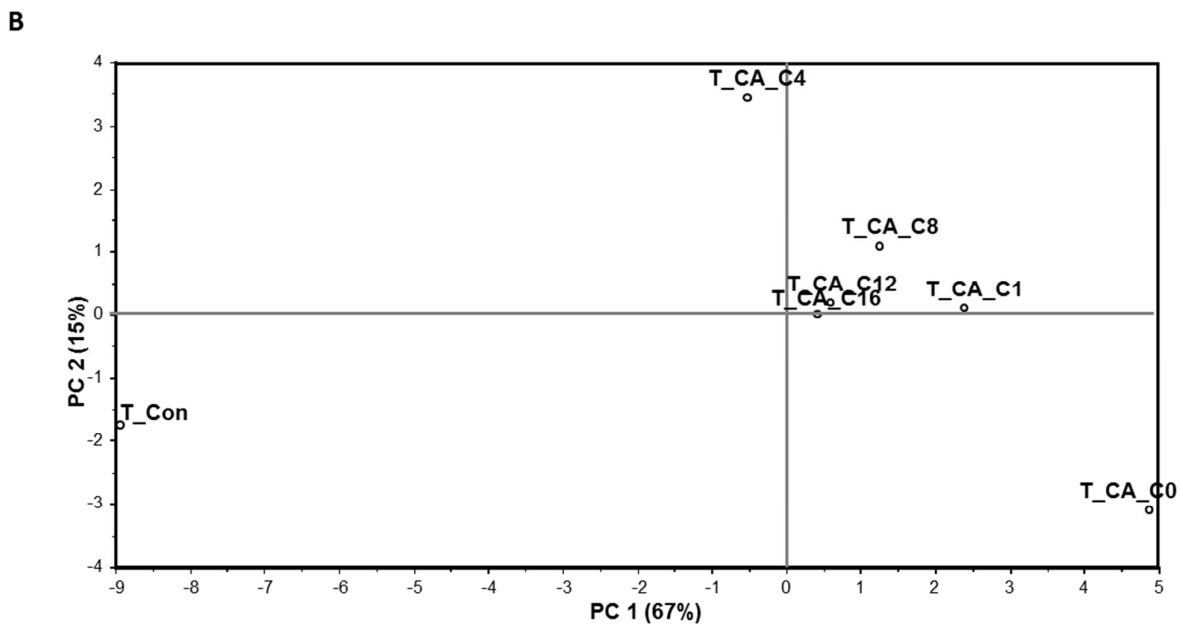
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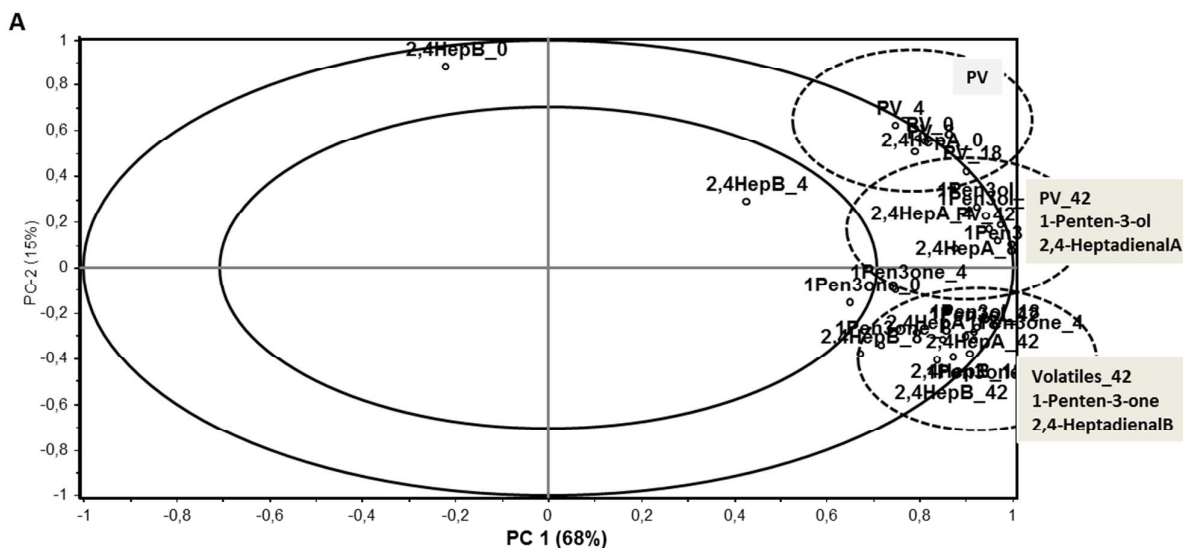


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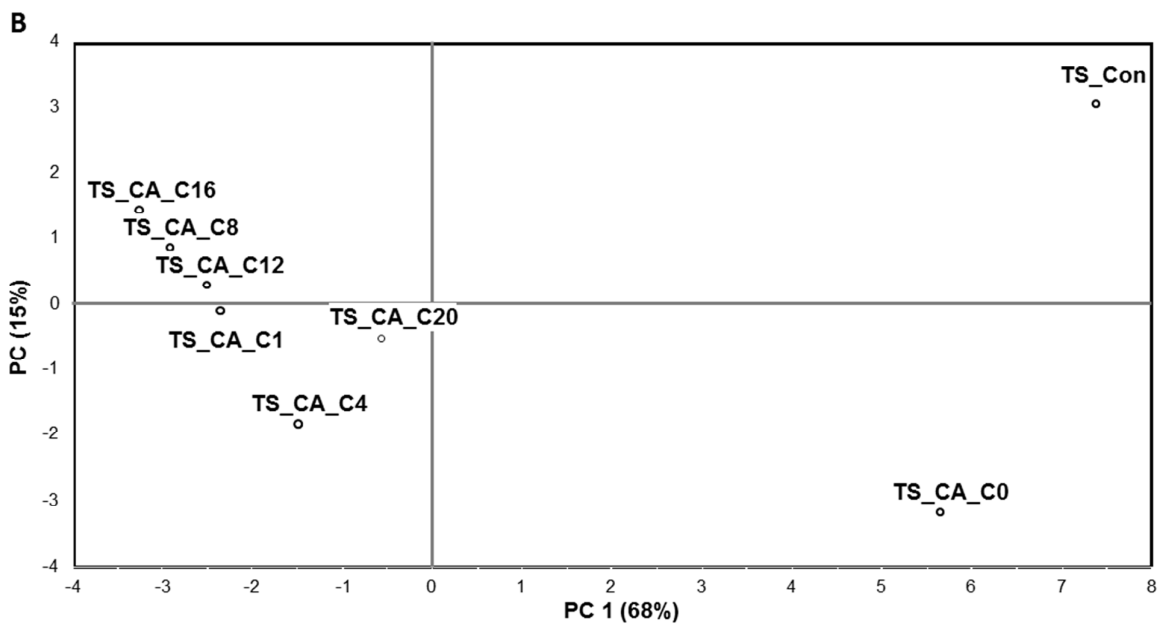
699 **Figure 2**

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704 **Figure 3**

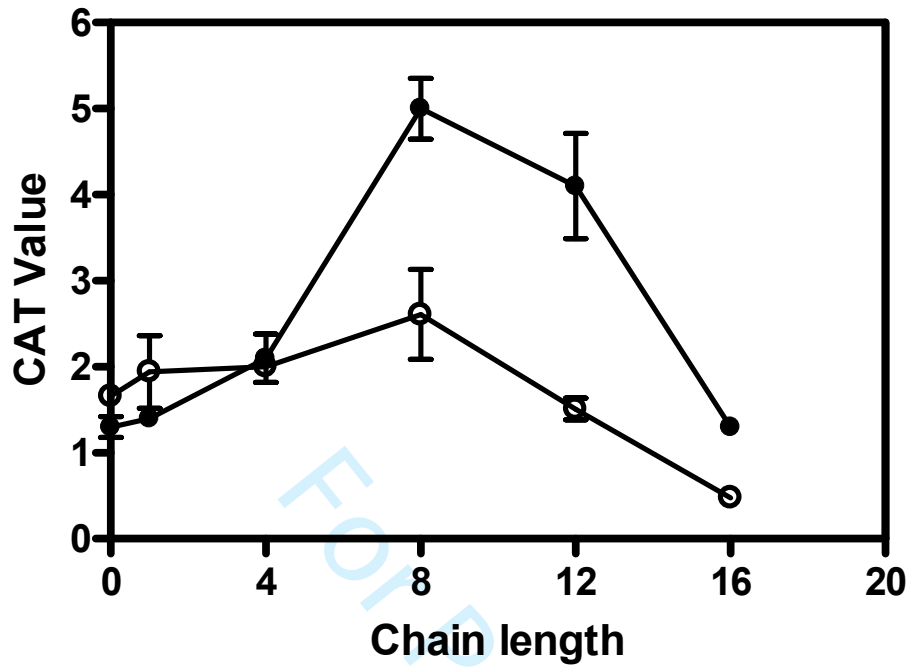


Figure 4

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8 1 | Alkyl caffeates as antioxidants in ~~e/w~~O/W emulsions: Impact of emulsifier type and endogenous  
9 2 | tocopherols

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14 4 | Ann-Dorit Moltke Sørensen<sup>1\*</sup>, Pierre Villeneuve<sup>2</sup>, Charlotte Jacobsen<sup>1</sup>

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29 11 | Running title: ~~Oxidation is affected by emulsifier and antioxidant~~ Effect of emulsifier and  
30 12 | antioxidants on lipid oxidation

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33  
34 13 | Keywords: Caffeic acid; Lipid oxidation; Tween; Citrem; Phenolipids; Fish oil; Antioxidant  
35 14 | interactions

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38 15  
39  
40 16 | Abbreviations: CA Caffeic acid; C1 Methyl caffeate; C4 Butyl caffeate; C8 Octyl caffeate; C12  
41 17 | Dodecyl caffeate; C16 Hexadecyl caffeate; C20 Eicosyl caffeate; CAT Conjugated autoxidizable  
42 18 | triene; CCL Critical chain length; FO Fish oil; FO/RO Fish oil and rapeseed oil mixture (1:1); PC  
43 19 | Principal component; PCA Principal component analysis; PV Peroxide value; THF Tetrahydrofuran  
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**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 Tween<sup>80</sup>) 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

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

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## 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.



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

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## 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  $\alpha$ -tocopherol / kg, 98 mg  $\gamma$ -tocopherol / kg and 48 mg  $\delta$ -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  $\alpha$ -tocopherol / kg, 31 mg  $\beta$ -tocopherol / kg, 151 mg  $\gamma$ -tocopherol / kg and 6 mg  $\delta$ -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)

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8 122 dihydrochloride) and trolox were purchased from Sigma-Aldrich (Steinheim, Germany).  
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10 123 Synperonic was purchased from CRODA (East Yorkshire, UK). All solvents used were of HPLC  
11 124 grade and purchased from Lab-Scan (Dublin, Ireland). The external standards used for  
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13 125 quantification of secondary oxidation products were from Sigma Aldrich (Steinheim, Germany).  
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### 17 18 127 **Experimental design**

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20 128 For specific details on emulsifier, oil and antioxidants applied refer to Table 1. Experiment 1 was  
21  
22 129 designed to evaluate the effect caffeic acid and different alkyl caffeates as antioxidant in Citrem  
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24 130 stabilized emulsions in the presence of endogenous tocopherols due to the natural content of  
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26 131 tocopherols in oil. In this experiment fish oil was used, and a storage period of 15 days selected  
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28 132 based on previous storage experiment with fish oil.

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30 133 Experiment 2 was designed to evaluate the influence of endogenous tocopherols on the efficacy of  
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32 134 caffeic acid and caffeates in Tween stabilized emulsions. Tween was selected as emulsifier, since  
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34 135 Citrem contains tocopherols. Emulsions were prepared with and without endogenous tocopherols.  
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36 136 Thus, the oil without endogenous tocopherols was stripped (removal of tocopherols). Stripping of  
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38 137 fish oil will increase oxidation rates tremendously. To slow down oxidation rate, a mixture of fish  
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40 138 and rapeseed oil was therefore used instead in this experiment. The storage time was selected based  
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42 139 on preliminary laboratory trials (data not shown).

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44 140 Based on experiment 1 and 2 with endogenous tocopherols, the impact of emulsifier type is  
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46 141 evaluated despite different oil type and length of storage time.

### 47 48 142 49 50 143 **Removal of tocopherols from oils**

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8 144 FO/RO was stripped from tocopherols using an alumina packed glass column using hexane. For  
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10 145 further details refer to Sørensen et al [8]. The stripped oil was bottled, flushed with nitrogen and  
11 146 stored at -80°C until use for production of emulsions. Furthermore, the absence of tocopherols in  
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13 147 the oil was checked by HPLC according to the AOCS method [13]. After oil stripping (removal of  
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15 148 tocopherols) the PV was 0.5 meq. peroxides / kg oil and tocopherols were not detected.  
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### 19 150 **Production of ~~e/w~~O/W emulsions**

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22 151 Both in experiment 1 and 2, the emulsion compositions were 5% oil, 1% emulsifier and 94% 10  
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24 152 mM sodium acetate – imidazole buffer (pH 7). Antioxidants were diluted in methanol and added in  
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26 153 concentrations of 100 µM. For the control emulsions (without antioxidant added), methanol was  
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28 154 added in same amount as used for the methanolic antioxidant solutions added to the other  
29 155 emulsions. The short to medium chain phenolipids (C0 - C12) were added to the buffer (Citrem  
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31 156 emulsions) and buffer-emulsifier mixture (Tween emulsions), whereas the long chain phenolipids  
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33 157 (C16 – C20) were added to the oil-emulsifier mixture (Citrem emulsions) and oil (Tween  
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35 158 emulsions) before the pre-homogenisation step.  
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37 159 Preparation of emulsions for storage experiment 1 were produced with pre-emulsification (2 min,  
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39 160 Ultra-Turrax, Janke & Kunkel IKA-Labortechnik, Staufen, Germany) followed by homogenization  
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41 161 on a two-valve table homogenizer at a pressure of 800 bar (GEA Niro Soavi Spa, Parma, Italy). For  
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43 162 further details refer to Sørensen et al. [14]. Production of emulsions for storage experiment 2 were  
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45 163 pre-emulsified as in experiment 1, but homogenized on a microfluidizer (9K, Microfluidics,  
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47 164 Newton, MA, USA). Changes in the production between experiment 1 and 2 (homogenizer vs.  
48 165 microfluidizer) wasere done in order to operate with smaller emulsion volumes and; thereby reduce  
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50 166 the amount of phenolipids. Moreover, it became possible to, and the ability to cool the emulsion  
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8 167 during production which was an advantage due to the fact that ~~when the oil was stripped from~~  
9 tocopherols and would therefore be highly susceptible to oxidation during homogenization.  
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12 169 After production emulsions (100 g) were stored in 100 mL blue cap bottles at  $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . Samples,  
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14 170 one bottle pr. code, were taken at specific time points and divided into brown glass bottles, flushed  
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16 171 with nitrogen and stored at  $-40^{\circ}\text{C}$  until analyses, except for samples used to determine droplet size,  
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18 172 these samples were measured at the sampling day without pre-freezing.

### 19 20 173 **Droplet size measurements**

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23 174 Droplet size of the oil droplets in the ~~e/w~~O/W emulsion was determined by laser diffraction  
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25 175 (Mastersizer2000, Malvern Instruments Ltd., Worcestershire, UK). Few droplets of the different  
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27 176 emulsions were suspended directly in recirculating water (2800 rpm, obscuration 12-14%). Water  
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29 177 ( $\text{RI}_{\text{water}} = 1.330$ ) and sunflower oil ( $\text{RI}_{\text{oil}} = 1.469$ ) were used in this measurements as dispersant and  
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31 178 particle, respectively. Each sample was measured in triplicate ( $n=3$ ) and results are reported as  
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33 179 surface mean diameter,  $D_{3,2}$  [15].  
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### 37 181 **Lipid extractions from ~~e/w~~O/W emulsions**

38  
39 182 Peroxide value (PV) and tocopherol analyses are performed on lipid extracts. Thus, the lipids were  
40  
41 183 extracted from the emulsions prior to these analyses according to the method described by Bligh  
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43 184 and Dyer [16] using a reduced amount of solvent [17]. For each sample code two lipid extractions  
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45 185 were performed ( $n=2$ ).  
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### 50 187 **Tocopherols**

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8 188 Lipid extracts were evaporated under nitrogen, re-dissolved in heptane and analyzed by HPLC  
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10 189 (Agilent 1100 Series, Agilent Technology, Palo Alto, CA, USA) according to the AOCS Official  
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12 190 Method Ce 8-89 [13]. A silica column (Waters (Dublin, Ireland), 150mm, 4.6mm, 3 $\mu$ m silica film)  
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14 191 was used for separation of the tocopherol homologues. This analysis was performed in duplicate on  
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16 192 each lipid extract and results reported as  $\mu$ g tocopherol / g emulsion.  
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#### 20 194 **Primary oxidation products: Peroxide value (PV)**

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23 195 PVs in the lipid extracts were determined by colorimetric method based on formation of an iron-  
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25 196 thiocyanate complex. The colored complex was measured on a spectrophotometer at 500 nm  
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27 197 (Shimadzu UV1800, Shimadzu Scientific Instruments, Columbia, MD, USA) [18]. The analysis  
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29 198 was performed in duplicate and reported as meq peroxides / kg oil.  
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#### 33 200 **Secondary oxidation products: Volatiles**

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36 201 Volatiles were released from the ~~o/w~~o/w emulsion using dynamic headspace (45°C for 30 min,  
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38 202 nitrogen flow of 150 mL/min). Volatiles were then collected and trapped on Tenax GR packed  
39  
40 203 tubes. To avoid foam and thus water on the tubes, 4 mL of antifoam (Synperonic, conc. 8 g /L  
41  
42 204 water) was added to each sample prior to the collection. Trapped volatiles were desorbed using an  
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44 205 automatic thermal desorber (ATD-400, Perkin Elmer, Waltham, MA, USA). The transfer line of  
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46 206 the ATD was connected to a gas chromatograph (Agilent 5890, Palo Alto, CA, USA) with a mass  
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48 207 selective detector (HP 5972). Volatiles were separated on a DB1701 column (30m x ID 0.25mm x  
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50 208 1 $\mu$ m film thickness, J&W Scientific, Folsom, CA, USA). The initial temperature of the oven was  
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52 209 45°C, which was kept for 5 minutes and then gradually increased as follows: 45-55°C 1.5°C/min,  
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8 210 55-90°C 2.5°C/min, 90-220°C 12°C/min and kept at 220°C for 4 minutes. Calibration curves  
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10 211 prepared from external standards was used for quantification of different volatiles. In experiment 1  
11 212 (Table 1), different concentrations of external standard solutions were prepared and 1 µL was  
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13 213 placed in Tenax tubes and analyzed. In experiment 2 (Table 1), different concentrations of external  
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15 214 standard solutions were prepared and added to fresh emulsion without antioxidant. Volatiles were  
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17 215 collected in the same way as for samples. The 2,4-heptadienal external standard appears as two  
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19 216 peaks in the chromatogramme, these peaks are termed A and B. Each sample code was analyzed in  
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21 217 triplicate (n=3) and results reported as ng volatile / g emulsion.  
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## 25 219 **Partitioning**

26 220 Partitioning of caffeic acid and the different alkyl caffeates in buffer/oil, emulsifier/buffer and in  
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28 221 o/w/o/w emulsion was measured according to the method described by Schwarz et al. [19] with  
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30 222 modifications as described elsewhere. With this method it is assumed that the partitioning of the  
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32 223 antioxidants, equilibria reached, is not disrupted by centrifugation. For further details refer to  
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34 224 Sørensen et al. [20].  
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38 225 In short, the concentration of antioxidants was measured in the separated aqueous phase of 3  
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40 226 different systems: buffer / oil (FO or FO/RO), buffer / emulsifier (Citrem or Tween80) and 5%  
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42 227 o/w/o/w emulsions. Antioxidants were dissolved in methanol and added in a concentration of 100  
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44 228 µM. Separation of the aqueous phase was carried out 24 h after production of the different systems.  
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## 46 229 47 48 230 **Determination of concentration of added antioxidant**

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8 231 Caffeic acid and alkyl caffeates in the aqueous phases were analyzed by HPLC (Agilent 1100  
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10 232 Series, Agilent Technology, CA, USA) with a C18 Thermo Hypersil® ODS (250x4.6 mm, 5µ)  
11 233 column and using a gradient elution at a flow rate of 1 mL/min. Solvent A was 3 mM phosphoric  
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13 234 acid and solvent B was methanol. Gradient condition: 0-30 min 0-100% B, 30-40 min 100% B and  
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15 235 40-45 min 100-0% B. Injection volume was 20 µL. Caffeic acid and different alkyl caffeates were  
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17 236 quantified by calibration curves of these compounds dissolved in methanol.  
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### 19 237 20 21 22 238 **Conjugated autoxidizable triene (CAT) Assay**

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24 239 Stock solutions of the different compounds: caffeic acid, caffeates and trolox were prepared in  
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26 240 methanol. The CAT assay was performed as described in Sørensen et al. [8] with a modification.  
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28 241 This assay was performed with non-stripped tung oil, hence, the assay contained tocopherol and the  
29 242 evaluated antioxidant in a mixture. Each microplate well contained a microemulsion with the  
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31 243 following composition: 115 µM tung oil, 17 µM Brij 35, 1 mM AAPH and antioxidant (caffeic  
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33 244 acid, caffeates or Trolox) in various concentrations. The progress of lipid oxidation was followed by  
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35 245 measuring the decrease in absorbance at 273 nm. Each antioxidant concentration was measured in  
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37 246 triplicate on the plate and via independent measurements (two different microplates), n = 6. Results  
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39 247 were expressed as CAT value (mean ± SD). This method was developed by Laguerre et al. [21]. For  
40 248 further details about the calculations refer to Laguerre et al. [4, 21].  
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### 45 250 **Data treatment**

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47 251 **Statistics.** The results obtained were analyzed using one- and two-way ANOVA (GraphPad Prism,  
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49 252 Version 4.01, GraphPad Software Inc). Bonferroni multiple comparison post-test was used to  
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51 253 determine significant differences between samples or storage times. The significance level applied  
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was 95% ( $p < 0.05$ ). ~~When a significant 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:

$$\text{Inhibition [\%]} = \left( \frac{\text{Emulsion}_{\text{Control}} - \text{Emulsion}_{\text{Antioxidant}}}{\text{Emulsion}_{\text{Control}}} \right) \times 100$$

$\text{Emulsion}_{\text{Control}}$  and  $\text{Emulsion}_{\text{Antioxidant}}$  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 (D<sub>3,2</sub> (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

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8 297 octyl- (C8) and dodecyl caffeates (C12) were detected in the aqueous phase and the concentrations  
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10 298 were not significantly different from that of butyl caffeate (C4). The concentration of caffeic acid  
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12 299 and methyl caffeate in the aqueous phase was significantly lower when Tween was applied as  
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14 300 emulsifier than when Citrem was applied. Moreover, the concentration of butyl caffeate tended to  
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16 301 be lower (not significant) in the aqueous phase with Tween as emulsifier than with Citrem as also  
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18 302 observed for caffeic acid and methyl caffeate. These partitioning results indicated interactions  
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20 303 between antioxidants and emulsifier to a higher extent with Tween than Citrem, thus, resulting in a  
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22 304 higher concentration of antioxidant at the interface with Tween than Citrem as emulsifier. In  
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24 305 addition, the results indicated that there was a tendency, ~~however, not significant~~, to a lower  
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26 306 concentration of caffeic acid and methyl caffeate in the aqueous phase of the non-stripped Tween  
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28 307 80 emulsion, i.e. when endogenous tocopherol was present. ~~However, the differences were not~~  
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30 308 significant (Table 2).

### 33 310 **Efficacy of caffeic acid and caffeates in emulsions**

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35 311 The antioxidant efficacy of caffeic acid and caffeates were evaluated in the three different  
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37 312 emulsions shown in Table 1 and in a modified CAT assay (modification: non-stripped tung oil  
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39 313 applied) to investigate the effect of the presence of endogenous tocopherols in this assay. The  
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41 314 efficacy of caffeic acid and caffeates was evaluated from the measured lipid oxidation during  
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43 315 storage. Lipid oxidation was followed by measuring PV (primary oxidation product) and volatiles  
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45 316 (secondary oxidation products). Volatile oxidation products that increased during storage were  
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47 317 identified and quantified. The quantified volatiles were markers of lipid oxidation products, most of  
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49 318 them originated from oxidation of n-3 PUFAs.

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8 319 **Citrem stabilized emulsions with endogenous tocopherols present.** The PV in this experiment  
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10 320 increased to 20-35 meq. peroxides / kg oil depending on the antioxidant treatment during the 15  
11 321 days of storage (data not shown). The concentration of volatiles after 15 days of storage  
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13 322 depended upon the specific volatile quantified and the antioxidant applied (1-penten-3-one 20-  
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15 323 60 ng / g emulsion, 1-penten-3-ol 100-300 ng / g emulsion, hexanal 70-150 ng / g emulsion, 4-  
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17 324 heptenal 10-25 ng / g emulsion, 2,4-heptadienal 4000-6000 ng / g emulsion and nonanal 30-70 ng /  
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19 325 g emulsion).

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21 326 A PCA of the PVs,  $\alpha$ -tocopherol and volatile compounds measured during storage explained 71%  
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23 327 of the variation in the obtained results by the first two principal components (PCs), Figure 1. The 3  
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25 328 other tocopherols ( $\beta$ -,  $\delta$ -, and  $\gamma$ -tocopherols) were excluded from the PCA model due to no or minor  
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27 329 changes during storage in the different emulsions. Figure 1A shows the correlation loadings i.e.  
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29 330 graphical mapping of the measured variables. Generally, all the volatiles were located in quadrant 1  
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31 331 (top-right part) and 4 (bottom-right part), PVs in quadrant 2 (top-left part) closer to PC 2 and  $\alpha$ -  
32 332 tocopherols in quadrant 2 and 3 (bottom-left part) a long PC 1. Thus, the first PC clearly described  
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34 333 lipid oxidation with tocopherol in the left side and volatiles in the right side. Comparing Figure 1A  
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36 334 with 1B, the scores, reveals the differences in efficacy between caffeic acid and caffeates. Butyl  
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38 335 caffeate was prooxidative due to its location in the 4th quadrant i.e. high concentration of many of  
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40 336 the volatile compounds. Octyl caffeate also acted as a prooxidant, however, not as strong as butyl  
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42 337 caffeate. Furthermore, hexadecyl caffeate's location in first quadrant, but close to PC 2, indicates a  
43 338 slight prooxidative effect. Dodecyl and eicosyl caffeates seemed to have no effect due to their  
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45 339 proximity to the control emulsion in the 2nd quadrant. The location of caffeic acid in the 3 quadrant  
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47 340 of the scores plot away from the volatile compounds (1 and 4 quadrant) indicates that this  
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49 341 antioxidant was the most efficient followed by methyl caffeate. However, caffeic acid and methyl  
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51 342 caffeate were not strong antioxidants, since some of the measured volatiles were present in higher

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8 343 concentration in these emulsions than in the control emulsion (Table 3, Inhibition percentages, Raw  
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10<sup>344</sup> data not shown).

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12<sup>345</sup> ***Tween stabilized emulsions with endogenous tocopherols present.*** In the Tween stabilized  
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14<sup>346</sup> emulsion with endogenous tocopherols present, the PV increased to 5-30 meq. peroxides / kg oil  
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16<sup>347</sup> depending on the antioxidant treatment during the 6 days of storage. The concentration of volatiles  
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18<sup>348</sup> after 6 days of storage dependent upon the specific volatile quantified and the antioxidant applied  
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20<sup>349</sup> (1-penten-3-one 2-10 ng / g emulsion, 1-penten-3-ol 5-30 ng / g emulsion and 2,4-heptadienal 50-  
21<sup>350</sup> 350 ng / g emulsion).

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24<sup>351</sup> A PCA of the PVs,  $\alpha$ -tocopherol and volatile compounds (1-penten-3-one, 1-penten-3-ol and 2,4-  
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26<sup>352</sup> heptadienal) measured during storage explained 82% of the variation in the obtained results by the  
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28<sup>353</sup> first two PCs (Figure 2). Similar to the other PCA model on Citrem stabilized emulsions, the 3 other  
29<sup>354</sup> tocopherol homologues ( $\beta$ -,  $\delta$ -, and  $\gamma$ -tocopherols) were excluded from the PCA model due to no or  
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31<sup>355</sup> minor changes during storage in the different emulsions. The PV and all the volatiles were located  
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33<sup>356</sup> to the left in the plot of the correlation loadings (Figure 2A). Tocopherols were located opposite to  
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35<sup>357</sup> the PV and volatiles. From the correlation loadings plot it is clear that the first PC describe lipid  
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37<sup>358</sup> oxidation with tocopherol in the right side and volatiles in the left side, thus, increased lipid  
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39<sup>359</sup> oxidation moving from the right to the left in the plot. Scores plot (Figure 2B) reveals differences in  
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41<sup>360</sup> efficacy between caffeic acid and caffeates in the tween stabilized emulsions. The control emulsion  
42<sup>361</sup> was located to the left side of the PC 2 axis in the scores plot and all emulsions with antioxidant  
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44<sup>362</sup> added were located to the right of the PC 2 axis except emulsion with butyl caffeate added (Figure  
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46<sup>363</sup> 2B). In connection with correlation loadings plot, this indicates that all the emulsions with  
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48<sup>364</sup> antioxidant added acted as antioxidant in the tween stabilized emulsions. As described above, butyl  
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50<sup>365</sup> caffeate was located opposite to the PC 2 axis compared to the other emulsions with antioxidant  
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52<sup>366</sup> added, this is explained by the higher amount of 2,4-heptadienal at day 6 in this emulsion (Figure

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8 367 2). Butyl caffeate worked as antioxidant for all other oxidation parameters measured; however, it  
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10 368 was the least efficient due to higher amount of the measured oxidation parameters compared to the  
11 369 other antioxidant applied (Table 3). Moreover, the PCA model reveals that caffeic acid without  
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13 370 esterification was more efficient in tween stabilized emulsions followed by methyl caffeate (short  
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15 371 chain esterification) due to their location most far away from the oxidation parameters measured.  
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17 372 Raw data supported the PCA model.

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19 373 ***Tween80 stabilized emulsions without endogenous tocopherols present.*** In the Tween stabilized  
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21 374 emulsion with endogenous tocopherols present, the PV increased ~~to from 08- to~~ 14 meq. peroxides  
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23 375 / kg oil ~~without antioxidant added depending on the antioxidant treatment~~ during the 42 hours of  
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25 376 storage; ~~whereas, the PV increased from 2-to 8 and 1- to 4 meq. Peroxides / kg oil with caffeic~~  
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27 377 ~~acid and eicosyl caffeates, respectively. The other antioxidant treatments resulted in no increase in~~  
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29 378 ~~PV during storage.~~ -The concentration of volatiles after 42 hours of storage dependent upon the  
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31 379 specific volatile quantified and the antioxidant applied (1-penten-3-one 0-3 ng / g emulsion, 1-  
32 380 penten-3-ol 0-9 ng / g emulsion and 2,4-heptadienal 2-90 ng / g emulsion), which was much lower  
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34 381 than in the Citrem stabilized emulsions.

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37 382 A PCA of the PVs and volatile compounds (1-penten-3-one, 1-penten-3-ol, ~~4 heptenal~~ and 2,4-  
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39 383 heptadienal) measured during storage explained 83% of the variation in the obtained results by the  
40 384 first to principal components (PCs), Figure 3. All the volatiles were located in quadrant 1 (~~top-right~~  
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42 385 ~~part~~) and 4 (~~bottom-right part~~). The first PC clearly describes lipid oxidation (right side) versus no  
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44 386 lipid oxidation (left side), whereas, PC 2 describes the development of lipid oxidation over time,  
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46 387 with PV and volatiles in the beginning of the storage period located in the top of the plot and in the  
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48 388 bottom of the plot after 42 days~~here increasing oxidation has decreasing PC 2 values~~ (Figure 3A).  
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50 389 Comparing Figure 3A with 3B, the scores plot, it is observed that control emulsion and emulsion  
51 390 with caffeic acid is located in the same side as the oxidation parameters measured. Thus, caffeic  
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8 391 acid is acting as a prooxidant in Tween stabilized emulsions without endogenous tocopherol  
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10 392 present. Esterification of caffeic acid in Tween stabilized emulsion without tocopherols improved  
11 393 its antioxidative properties, since all caffeates evaluated were acting as antioxidants. However, it  
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13 394 seems like caffeic acid esterified with C20 was slightly less efficient than the other esters evaluated  
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15 395 (Table 3). Raw data supports the observation from the PCA model (data not shown).  
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18 396 ***Comparison of the influence of emulsifier type - Citrem versus Tween<sup>80</sup> stabilized emulsions.***

19 397 Table 3 shows calculated inhibition percentages for caffeic acid and caffeates. It is clear that the  
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21 398 emulsifier impacted the efficacy of the antioxidants added. In Citrem stabilized emulsions, caffeic  
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23 399 acid and methyl caffeate were the only ones acting as antioxidants. Their antioxidative effect in this  
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25 400 model emulsion was weak to intermediate and they even promoted the formation of certain  
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27 401 volatiles. In contrast, caffeic acid and all the evaluated caffeates in Tween stabilized emulsions  
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29 402 acted as antioxidants. Caffeic acid was the strongest antioxidant followed by methyl caffeate.  
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31 403 ***Comparison of the influence of the presence of endogenous tocopherols.*** Calculated inhibition  
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33 404 percentages for selected oxidation variables measured in Tween stabilized emulsions with and  
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35 405 without endogenous tocopherols are presented in Table 3. A clear difference in the efficacy of  
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37 406 antioxidants was that caffeic acid acted as a strong antioxidant when tocopherols were present,  
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39 407 whereas, it acted as intermediate to strong prooxidant without tocopherols in this model emulsion.  
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41 408 In addition, the caffeates acted as stronger antioxidants without tocopherols compared to the same  
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43 409 model emulsion with tocopherols present (Table 3). Caffeates with chain lengths between C1 and  
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45 410 C18 were all strong antioxidants, whereas the antioxidant efficacy was decreased when the chain  
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47 411 length was increased to C20.

48 412 ***Antioxidant efficacy in a modified CAT assay.*** The CAT assay is an assay developed to measure  
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50 413 the efficacy of antioxidants in a micro emulsion system without endogenous tocopherols present.  
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8 414 The assay was slightly modified to investigate the efficacy of caffeic acid and caffeates in the  
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10 415 presence of endogenous tocopherols. The results are shown in Figure 4 together with results  
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12 416 obtained earlier with an unmodified CAT assay [8]. When tocopherol was present the antioxidative  
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14 417 efficacies of caffeic acid, methyl-, butyl-, octyl- and dodecyl caffeates were not significantly  
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16 418 different. This finding is different from earlier results obtained without the presence of tocopherols,  
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18 419 where octyl- and dodecyl caffeates exerted a significantly higher efficacy than caffeic acid and the  
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20 420 other caffeates evaluated. Furthermore, the efficacy of octyl- and dodecyl caffeates without  
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21 421 tocopherols present was also significantly higher than when tocopherols were present.  
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## 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 ~~Only~~ 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

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8 446 changing emulsifier affects the partitioning of the antioxidants and the resulting antioxidant activity  
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10 447 measured in emulsion systems [26]. The different emulsifier evaluated was SDS, CTAB, Brij58 and  
11 448 PHLC, and their partitioning study revealed increased solubility effect of the emulsifiers in the  
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13 449 following order: PHLC < SDS < Brij58 < CTAB. The antioxidant activity of gallic acid and ethyl  
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15 450 gallate based on the formation of hydroperoxides and hexanal increased in the following order:  
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17 451 CTAB (no activity measured) < Brij58 < PHLC < SDS. Gallic acid only showed antioxidant  
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19 452 activity with PHLC stabilized emulsions. This was a reverse order compared to the partitioning  
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21 453 measured. Hence, it was suggested that the increased partitioning into the emulsifier layer and lipid  
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23 454 counteract the hydrogen-donating ability, and lower the activity of the antioxidants [246].  
24 455 Moreover, Pekkarinen et al. [257] evaluated antioxidative effect and partitioning of phenolics in  
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26 456 different systems. Interaction between caffeic acid and Tween 20 differed from other phenolics such  
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28 457 as vanillic acid, ferulic acid and sinapic acid evaluated, since Tween 20 exhibited higher  
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30 458 solubilisation capacity for caffeic acid than for other phenolic acids. Additionally, Pekkarinen et al.  
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32 459 [257] concluded that these antioxidant-emulsifier interactions have a strong influence on the  
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34 460 partitioning of antioxidants. The partitioning results obtained in this study confirmed that Citrem  
35 461 and Tween as emulsifiers results in differences in the partitioning of caffeic acid and caffeates. Less  
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37 462 caffeic acid and caffeates were present in the aqueous phase when Tween was applied. This clearly  
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39 463 demonstrated stronger antioxidant-emulsifier interaction with Tween compared to Citrem.  
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41 464 Moreover, Citrem is an anionic emulsifier, thus, the interface is negatively charged and will repel  
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43 465 negatively charged antioxidants i.e. caffeic acid, which could explain why caffeic acid interacted  
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45 466 less with Citrem than with Tween. An explanation for the stronger interactions with Tween may be  
46 467 the molecular structure of the emulsifiers, since Tween is a larger and more bulky molecule than  
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48 468 Citrem; however, this has to be further evaluated. Furthermore, Schwarz et al. [19, 268, 279]  
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50 469 evaluated partitioning of different antioxidants in dispersed lipid systems with different emulsifiers.

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8 470 Significant differences were observed in partitioning of the antioxidants between phases, both as a  
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10 471 function of pH and emulsifier type and concentration. It was concluded from the results that  
11 472 determination of antioxidant partitioning may be an important tool to select antioxidants structurally  
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13 473 designed to localize at the surfaces [19], however, the partitioning of the antioxidants cannot alone  
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15 474 explain the measured antioxidant activity in emulsions [279]. Results from another study by  
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17 475 Schwarz et al. [2830] evaluating antioxidant activity of antioxidants with different lipophilicity in  
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19 476 bulk oil, ~~o/w~~O/W and ~~w/o~~W/O emulsions with different emulsifiers led to the assumption that  
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21 477 differences in antioxidant activity for the same emulsion type might be additionally influenced by  
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23 478 interaction with the emulsifier dominating the interfaces in the emulsion system [2830]. The  
24 479 obtained results for the two emulsifiers together with the partitioning study may also here lead to  
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26 480 the assumption that emulsifier-antioxidant interactions (e.g. hydrogen bonding) affected the  
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28 481 antioxidant activity of the caffeates. However, the type of interactions and the impact of  
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30 482 antioxidant-emulsifier interactions on the radical scavenging activity have to be studied more in  
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32 483 more details to make further conclusions.

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34 484 ***Impact of endogenous tocopherols on the efficacy of caffeic acid and caffeates.*** The presence of  
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36 485 endogenous tocopherol not only changed the antioxidant activity of caffeic acid and caffeates in  
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38 486 both the storage experiment and in the CAT assay, but also their partitioning in the emulsion  
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40 487 system. A tendency to less caffeic acid and caffeates (C1, C4 and C12) present in the aqueous phase  
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42 488 with endogenous tocopherol in the emulsion system was observed. This may indicate some  
43 489 interactions between tocopherol and caffeic acid / caffeates both for the antioxidative effect and  
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45 490 localization in the emulsion system. The use of a combination of antioxidants to produce synergistic  
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47 491 interaction has been reported earlier e.g. tocopherol regeneration by ascorbic acid, polyphenols and  
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49 492 flavonoids [2931-324]. Panaya et al. [324] carried out the only study investigating interactions  
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51 493 between tocopherol and a phenol (rosmarinic acid) and its alkyl esters (rosmarinates, C4, C12 and

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8 494 C20) in Tween20 stabilized emulsions. Rosmarinic acid exhibited strongest synergistic interaction  
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10 495 with tocopherol, and C4 and C12 esters exhibited small synergistic interaction. An antagonistic  
11 496 interaction was observed with C20 ester and tocopherol. Thus, the more hydrophilic rosmarinic acid  
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13 497 exhibited more interactions with the tocopheryl radical than the esters. In the present study, the  
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15 498 emulsion with the more hydrophilic caffeic acid exhibited better oxidative stability than the  
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17 499 emulsions with the esters (more hydrophobic antioxidants) when tocopherol was present as also  
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19 500 observed with rosmarinic acid and rosmarinates. Actually, caffeic acid turned from being  
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21 501 prooxidative without tocopherol present to being the most efficient antioxidant with endogenous  
22 502 tocopherol present in Tween stabilized emulsion. In emulsions, the majority of the emulsifier is  
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24 503 accumulated at the oil-water interface. However, a part of the emulsifier is not associated with the  
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26 504 oil-water interface if the emulsifier concentration is above the CMC (critical micellar concentration,  
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28 505 CMC Tween80 13-15 mg/L, Sigma) and will form micelles in the aqueous phase. In this study, the  
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30 506 concentration of Tween was much higher than CMC (10 g/L). The decreased antioxidant efficiency  
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32 507 of the different caffeates compared with caffeic acid is suggested to be due to the solubilisation of  
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34 508 caffeates in Tween micelles, thus, localized away from the interface unable to inhibit lipid  
35 509 oxidation. Although, micelles are not isolated structures, they compromise structures that are in  
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37 510 dynamic equilibrium with other structures in the emulsion system. This means that components can  
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39 511 be exchanged between the different structures i.e. between micelles and emulsion droplets [35, 36].  
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41 512 Thus, more studies are needed to further elucidate the differences observed in partitioning and  
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43 513 antioxidant effect in the presence of endogenous tocopherols.

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45 514 Additionally, it is assumed that caffeic acid is located in close proximity to the interface where it  
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47 515 regenerates tocopherol at the interface in spite of the repelling effect of two negatively charged  
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49 516 compounds (Citrem and caffeic acid). The proposed partial location of tocopherol at the interface is  
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51 517 supported by Jacobsen et al. [37] who reported that ca. 6 % of the alpha-tocopherol present in

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8 518 mayonnaise was located at the interface whereas the remaining tocopherol was located in the oil  
9 phase. Therefore, lipophilization is not needed to improve the oxidative stability of these emulsions,  
10 519  
11 520 due to tocopherols location at the interface.  
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14 521 ***Antioxidant hypotheses and efficacies of caffeic acid and caffeates.*** The polar paradox was not  
15 confirmed in this study since caffeic acid was more efficient as antioxidant than caffeates in Citrem  
16 522 stabilized emulsions when endogenous tocopherol was present. In Tween stabilized emulsions with  
17 523 endogenous tocopherol caffeic acid followed by methyl caffeate were the most efficient  
18 524 antioxidants, whereas, caffeic acid acted as a prooxidant when tocopherol was not present. No cut-  
19 525 off effect was observed for the lipophilized caffeic acid, since the most efficient antioxidant in both  
20 526 Citrem and Tween stabilized emulsions when tocopherol was present was C0 (caffeic acid). A  
21 527 similar finding was observed for the CAT assay with endogenous tocopherol present. Without  
22 528 endogenous tocopherol present, the caffeates were most efficient antioxidants in Tween stabilized  
23 529 emulsions, a cut-off effect was found at C16. However, the efficiency of C20 was still an  
24 530 intermediate to strong antioxidant. The CAT assay showed a cut-off effect at around C8 and C12  
25 531 with no endogenous tocopherol present. The partitioning experiment clearly showed an effect of the  
26 532 chain length, with less antioxidant present in the aqueous phase with increasing antioxidant  
27 533 lipophilicity. Pekkarinen et al. [247] observed that the proportion of antioxidant solubilized in the  
28 534 lipid phase and particularly in the interface did not necessarily reflect the efficiency of the  
29 535 antioxidant. It was assumed from their evaluation of antioxidant activity and partitioning that  
30 536 specific interactions of the antioxidant with other compounds e.g. emulsifiers, and intermolecular  
31 537 hydrogen bonds may play an important role in reducing antioxidant activity. Furthermore,  
32 538 antioxidant-emulsifier interaction has a strong influence on partitioning of the antioxidant. It is  
33 539 suggested that the caffeates interacts with the emulsifier in form of micelles when endogenous  
34 540 tocopherols are present, resulting in reduced or no antioxidant activity. When endogenous  
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8 542 tocopherols are not present the caffeates is more likely to be solubilized at the interface instead of  
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10 543 the tocopherols. However, this has to be evaluated more in depth to conclude further on these  
11 544 differences in partitioning and antioxidant activity with the impact of endogenous tocopherols.  
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14 545 **Conclusions.** Partitioning and antioxidant activity of caffeic acid and caffeates were influenced  
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16 546 both by the emulsifier type and the presence of endogenous tocopherols. Thus, this study clearly  
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18 547 demonstrated different emulsifier-antioxidant and antioxidant-antioxidant interactions that affected  
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20 548 the efficacy of the evaluated caffeic acid and caffeates as antioxidant in emulsions. The hypotheses  
21 549 about antioxidant in emulsions are based on simple emulsions systems without the presence of  
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23 550 tocopherols. However, the impact of the presence of tocopherols on the efficacy of other  
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25 551 antioxidants is important since most food systems contain tocopherol.  
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13  
14 555 and Citrem, respectively.

15  
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17  
18 557 food systems – Effect of alkyl chain length and concentration” with project no 10-093655 financed  
19  
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23 559 The authors declare no conflicts of interest.

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78 664 **Figure legends**

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10 665 **Figure 1** PCA build on results obtained from PV, volatiles (1-penten-3-one, 1-penten-3-ol, 4-  
11 heptenal, 2,4-heptadienal, hexanal and nonanal) and  $\alpha$ -tocopherols measured on Citrem stabilized  
12 666 emulsions during storage (15 days) using full cross validation. A) Correlation loadings and B)  
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14 667 Scores plot. Abbreviations for sample codes refer to Table 1.  
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18 669 **Figure 2** PCA build on results obtained from PV, volatiles (1-penten-3-one, 1-penten-3-ol and 2,4-  
19 heptadienal) and  $\alpha$ -tocopherols measured on ~~tween-Tween80~~ stabilized emulsions during storage (6  
20 670 days) using full cross validation. A) Correlation loadings and B) Scores plot. Abbreviations for  
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22 671 sample codes refer to Table 1.  
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26 673 **Figure 3** PCA build on results obtained from PV and volatiles (1-penten-3-one, 1-penten-3-ol and  
27 2,4-heptadienal) measured on ~~tween-Tween80~~ stabilized emulsions during storage (42 hours) using  
28 674 full cross validation. A) Correlation loadings and B) Scores plot. Abbreviations for sample codes  
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30 675 refer to Table 1.  
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34 677 **Figure 4** CAT Value of caffeic acid and caffeates (C1-C16) measured in the concentration range of  
35 0.5 – 2  $\mu$ M. ● CAT Values determined without endogenous tocopherols (normal condition for the  
36 678 CAT assay, published in Sørensen et al. [8]) and ○ CAT Values determined with endogenous  
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38 679 tocopherols (modified CAT assay).  
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**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
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	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
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2	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)

686 **Table 2** Concentration [ $\mu\text{M}$ ] of caffeic acid and caffeates (Methyl, Butyl, Octyl and Dodecyl) measured in the aqueous phase of different  
 687 systems: 95% Buffer / 5% Oil, 99% Buffer / 1% Emulsifier and Emulsion (5% Oil, 1% Emulsifier and 94% Buffer). Citrem and Tween80  
 688 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 $\pm$ 3.1 <sup>a,b,x</sup>	91.5 $\pm$ 3.6 <sup>b,x</sup>	91.5 $\pm$ 7.1 <sup>b,x</sup>	93.7 $\pm$ 11.4 <sup>b,x</sup>	75.4 $\pm$ 7.0 <sup>c,x</sup>	74.0 $\pm$ 4.3 <sup>c,x</sup>	111 $\pm$ 2.6 <sup>a,x</sup>	78.4 $\pm$ 10.1 <sup>c,x</sup>
CA C1	86.2 $\pm$ 0.9 <sup>a,y</sup>	53.3 $\pm$ 3.0 <sup>b,y</sup>	43.8 $\pm$ 8.2 <sup>b,y</sup>	82.8 $\pm$ 1.9 <sup>a,x</sup>	15.4 $\pm$ 3.3 <sup>c,y</sup>	10.1 $\pm$ 2.5 <sup>c,y</sup>	91.1 $\pm$ 2.6 <sup>a,y</sup>	15.3 $\pm$ 3.1 <sup>c,y</sup>
CA C4	11.4 $\pm$ 0.4 <sup>a,b,z</sup>	4.09 $\pm$ 0.6 <sup>a,b,c,z</sup>	3.39 $\pm$ 0.5 <sup>b,c,z</sup>	11.3 $\pm$ 0.7 <sup>a,b,y</sup>	2.10 $\pm$ 0.3 <sup>c,z</sup>	< detection	13.0 $\pm$ 1.1 <sup>a,z</sup>	< detection
CA C8	< detection	< detection	< detection	< detection	2.60 $\pm$ 3.6 <sup>z</sup>	< detection	< detection	< detection
CA C12	< detection	< detection	< detection	2.00 $\pm$ 0.6 <sup>b,z</sup>	1.10 $\pm$ 0.4 <sup>z</sup>	< detection	9.70 $\pm$ 4.2 <sup>a,z</sup>	1.10 $\pm$ 0.2 <sup>b,y</sup>

689 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  
 690 c, whereas significant differences within a column i.e. same system but different antioxidant are denoted with x, y and z.

691 **Table 3** Calculated inhibition percentages for selected lipid oxidation parameters (PV, 1-penten-3-ol, 1-penten-3-one and 2,4-  
 692 heptadienalA) measured at selected time points in the different emulsions. Citrem stabilized emulsions are reported at day 6 and 15, Tween  
 693 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								

694 Abbreviation: AO Antioxidant; PV Peroxidase  $\Delta$ Value; 1Pen3ol 1-Penten-3-ol; 1Pen3one 1-Penten-3-one; 2,4HepA 2,4-HeptadienalA. [The 2,4-heptadienal external](#)  
 695 [standard appears as two peaks in the chromatogramme, these peaks are termed A and B \(here only 2,4HepA presented\).](#)



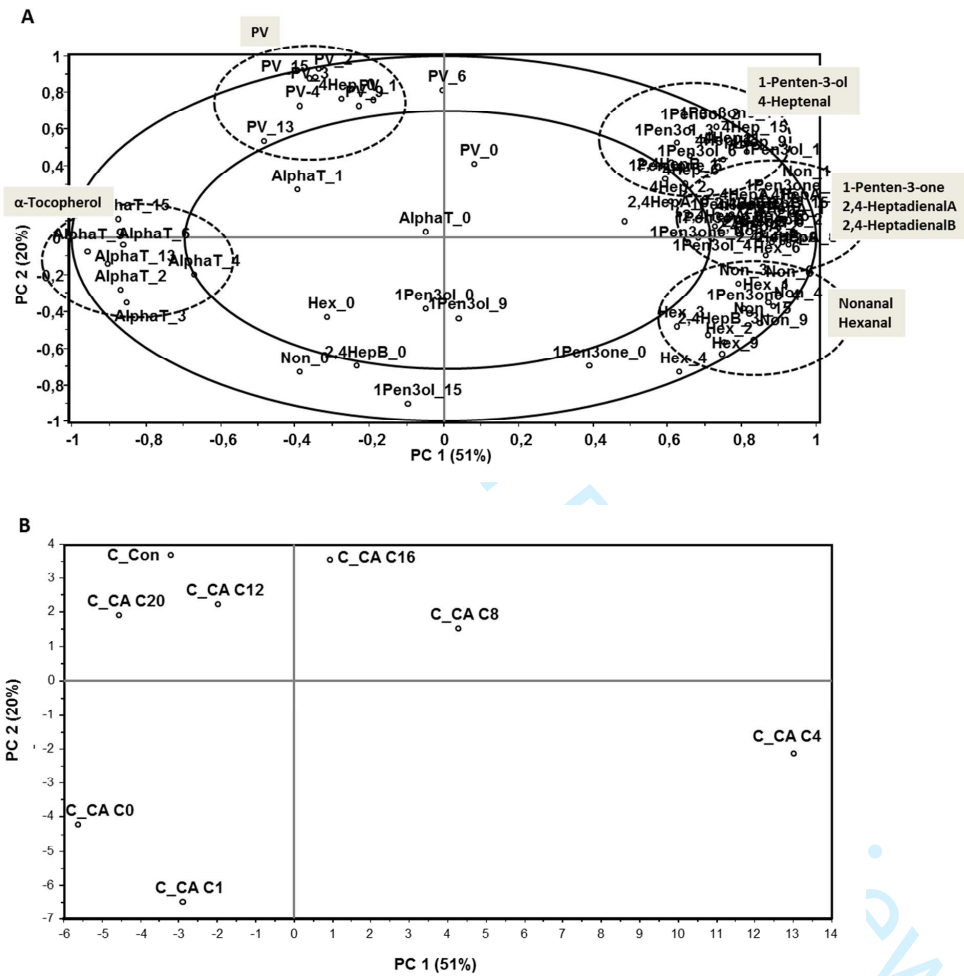


Figure 1

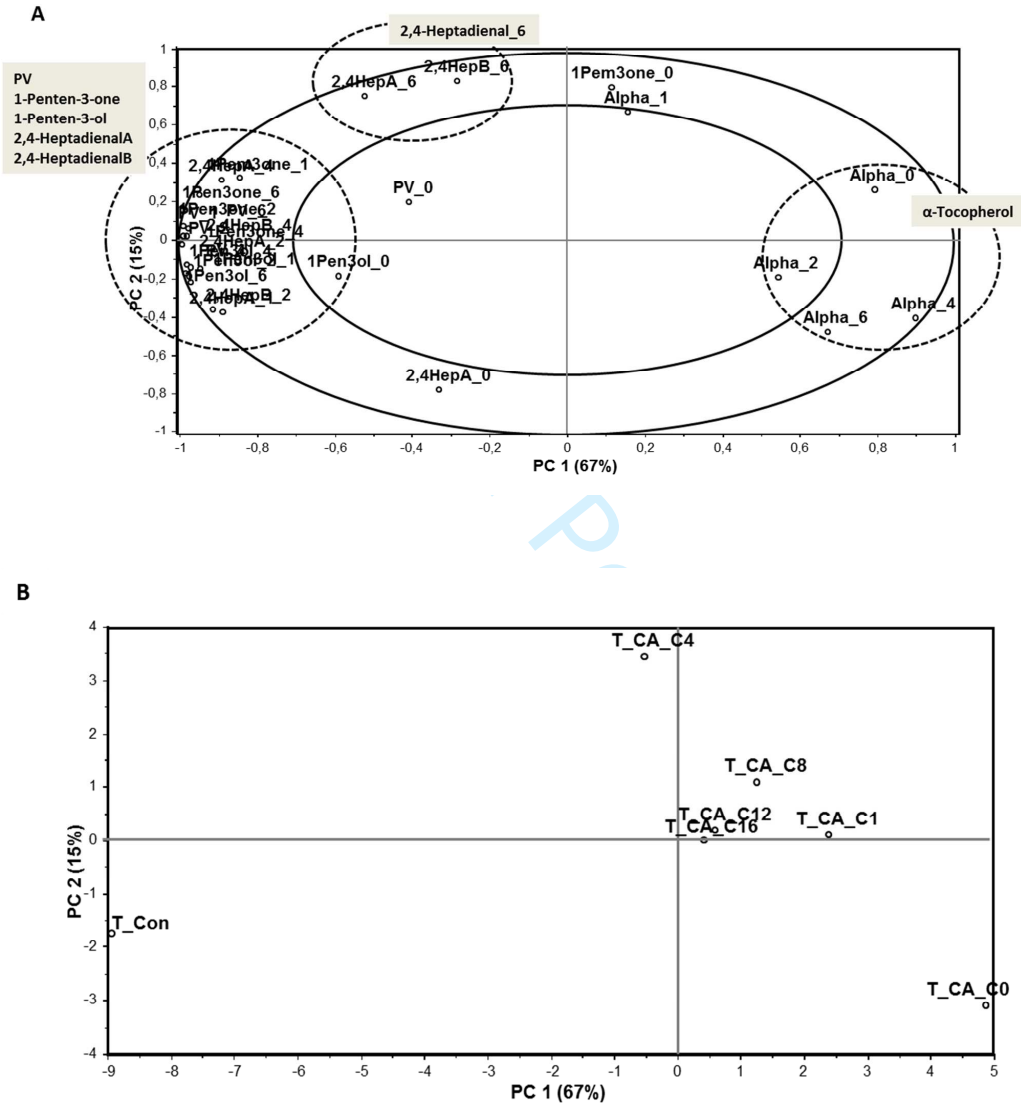


Figure 2

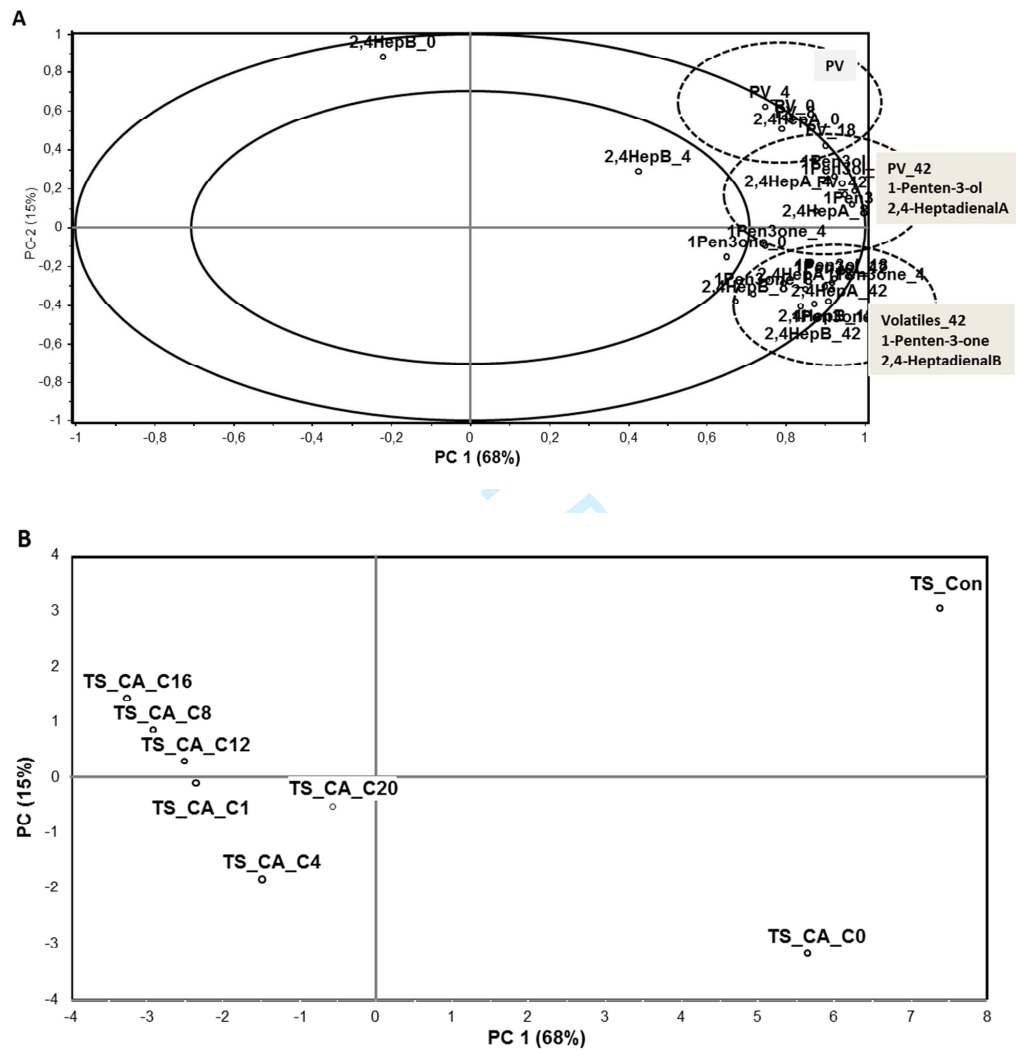


Figure 3

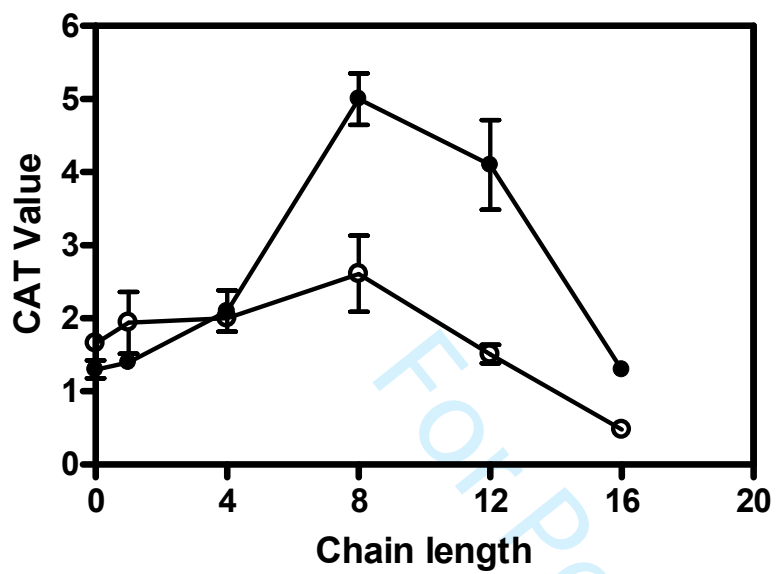
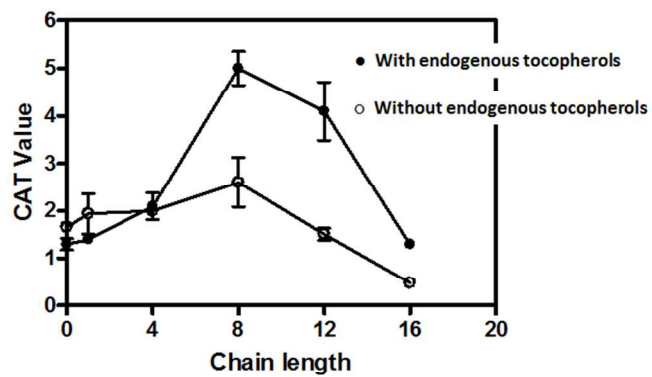


Figure 4

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4 **Graphical abstract.** CAT Value determined in oil-in-water micro emulsions with caffeic acid and  
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6 caffeates (100  $\mu\text{M}$ ) and with and without endogenous tocopherol and. Lipid oxidation was initiated  
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