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Interactions between Iron, Phenolic Compounds, Emulsifiers, and pH in Omega-3-Enriched Oil-in-Water Emulsions

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The behavior of antioxidants in emulsions is influenced by several factors such as pH and emulsifier type. This study aimed to evaluate the interaction between selected food emulsifiers, phenolic compounds, iron, and pH and their effect on the oxidative stability of n-3 polyunsaturated lipids in a 10% oil-in-water emulsion. The emulsifiers tested were Tween 80 and Citrem, and the phenolic compounds were naringenin, rutin, caffeic acid, and coumaric acid. Lipid oxidation was evaluated at all levels, that is, formation of radicals (ESR), hydroperoxides (PV), and secondary volatile oxidation products. When iron was present, the pH was crucial for the formation of lipid oxidation products. At pH 3 some phenolic compounds, especially caffeic acid, reduced Fe^{3+} to Fe^{2+}, and Fe^{2+} increased lipid oxidation at this pH compared to pH 6. Among the evaluated phenols, caffeic acid had the most significant effects, as caffeic acid was found to be prooxidative irrespective of pH, emulsifier type, and presence of iron, although the degrees of lipid oxidation were different at the different experimental conditions. The other evaluated phenols were prooxidative at pH 3 in Citrem-stabilized emulsions and had no significant effect at pH 6 in Citrem- or Tween-stabilized emulsions on the basis of the formation of volatiles. The results indicated that phenol−iron complexes/nanoparticles were formed at pH 6.

KEYWORDS: Lipid oxidation; emulsifiers; oil-in-water emulsions; phenolic compounds; antioxidative properties; pH

INTRODUCTION

Due to the health beneficial effects of n-3 polyunsaturated fatty acids (n-3 PUFA) there has been an increasing industrial interest in using marine oils in foods during the past decade. However, the use of marine oils in foods is limited by their oxidative susceptibility. Lipid oxidation is particularly a problem when marine oils are emulsified into various food systems as the emulsification process will lead to the formation of a large interfacial area and lipid oxidation has been suggested to be initiated at the interface between oil and water (*1, 2*). In recent decades, special attention has been given to the use of natural antioxidants, for example, phenolic compounds and carotenoids, for the prevention of oxidation because of the worldwide trend to avoid or minimize the use of synthetic food additives. Phenolic compounds such as caffeic acid and coumaric acid have received increasing interest due to their potential antioxidant activity. These compounds may exert their antioxidative effect by donating a hydrogen atom to free radicals, thereby acting as chain-breaking antioxidants, or they may act as antioxidants by metal chelation, which reduces the activity of prooxidants (*3–6*).

The activity of the phenolic compounds as antioxidants in food systems depends not only on the structure (i.e., number and position of hydroxyl groups bound to the aromatic ring) and chemical reactivity of the phenolics but also on other factors such as their physical location, other food components, and environmental conditions, for example, pH (*2, 7–9*).

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The behavior of antioxidants in emulsions is more complex than that in bulk oil. An emulsion is a multiphase system with different chemical environments in the different phases. Surface-active molecules, which may include reactive species as well as antioxidants, accumulate at the oil/water interface, giving comparably high concentrations. Hence, in the emulsion system several factors may influence lipid oxidation, for example, the emulsifier type (*10*). A change in pH alters the charge of the applied antioxidant and might also alter the charge of the emulsifier depending on its character (anionic, cationic, or nonionic). These changes may influence the location of the antioxidants because of repulsive or attractive forces between antioxidant and emulsifier and thereby also influence the efficacy of the antioxidants (*10–12*). Moreover, other properties of the emulsifier such as its structural properties and its ability to form hydrogen bonds or hydrophobic interactions with the antioxidant will also affect the activity of the antioxidants (*13*). Recent findings suggested that a close proximity of free radicals and antioxidants is a crucial prerequisite for the radical reducing action of antioxidants and that segregation of the antioxidant from the radicals by certain emulsifiers may prevent it from exerting its action (*14*). However, only a few studies have been carried out investigating the efficacies of antioxidants in emulsions containing emulsifiers relevant for food production. Likewise, the interaction between the emulsifiers, phenolic compounds, and pH has been investigated to only a limited extent.

Previous studies of oxidation in n-3 PUFA enriched food emulsions have shown that the efficiency of the antioxidant systems strongly depends on the distribution of the antioxidants between the continuous and dispersed phase. A general phenomenon, referred to as the "polar paradox", appears to exist with respect to the efficacy of the antioxidants in bulk oil and in dispersed systems (*15*). Polar antioxidants are more effective in bulk oil, whereas predominately nonpolar antioxidants are more effective in emulsions (*1, 8, 15*).

Phenolic compounds are predominately polar compounds and are expected to be in the water phase of an emulsion. However, the physical environment can change the partitioning of the phenolic compounds into the emulsifier pseudophase of an emulsion (*16*). Thus, our key hypothesis is that the emulsifier might change the distribution of the phenolic compounds and of iron, and thereby their effect on lipid oxidation in an emulsion system, and that the effect of the emulsifier on the antioxidant partitioning and on iron distribution may also be dependent on pH. Therefore, this study evaluated the effect of the interaction between selected food emulsifiers, phenolic compounds, and pH on the oxidative stability in a 10% oil-in-water emulsion (fish oil and medium-chain triglycerides, 1:1) at pH 3 and 6. The emulsifiers tested were polyoxyethylene sorbitan oleate (Tween 80) and a citric acid ester of a mono- and diglyceride (Citrem). Tween is a water-soluble, micelle-forming, nonionic emulsifier, whereas Citrem is a water-dispersible, but poorly soluble, anionic emulsifier. The selected phenolic compounds were naringenin, rutin, caffeic acid, and coumaric acid, which were evaluated in a concentration of 100 mg/kg. The oxidative stability of the emulsions was evaluated in two storage experiments, which had the following aims. Experiment 1 aimed to evaluate the interactions between iron, pH, and three phenolic compounds (naringenin, rutin, and caffeic acid) with very different structures and solubilities. Experiment 2 aimed to evaluate the interaction between iron, two structurally different emulsifiers, and two phenolic compounds (caffeic acid and coumaric acid) with relatively similar structures. In addition to the storage experiments, the antioxidant–iron interactions as well as the adsorption of the antioxidant on the emulsifier surface were evaluated. The structures of the different phenolic compounds and emulsifiers are illustrated in **Figure 1**.

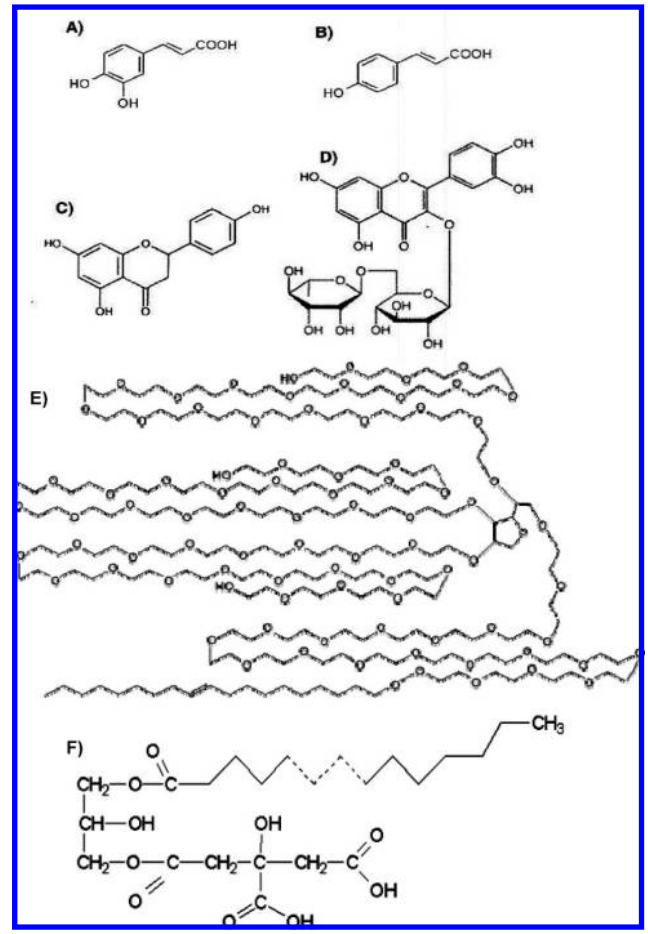

Figure 1. Structures of the phenolic compounds tested as antioxidants and the emulsifiers evaluated in the experiments: (**A**) caffeic acid; (**B**) *p*-coumaric acid; (**C**) naringenin; (**D**) rutin; (**E**) Tween; (**F**) Citrem.

MATERIALS AND METHODS

Materials. Medium-chain triglyceride (MCT, chain length C_6-C_{12}, whereof C_8 56% and C_{10} 43%, 99.3% triglycerides) Miglyol 812 was supplied from Sasol Germany GmbH (Witten, Germany). Commercial fish oil was supplied by Maritex A/S (Sortland, Norway) and stored at –40 °C until use. The fish oil was a cod liver oil, and the fatty acid composition was as follows: 14:0, 3.6%; 16:0, 10.3%; 18:0, 2.2%; 16:1, 6.4%; 18:1, 21.4%; 20:1, 11.3%; 22:1, 8.3%; 18:2, 1.7%; 18:3, 0.9%; 18:4, 2.5%; 20:5, 8.0%; 22:5, 1.0%; 22:6, 10.9%. The peroxide value, free fatty acids, and tocopherol content were 1.96 mequiv of peroxides/kg of oil, 0.06% free fatty acids, and 329 mg of α-tocopherol/kg, respectively. According to the specifications from the fish oil producer, the anisidine value was below 3.0. PV and tocopherol levels of the mixture of fish oil and MCT were approximately half of the values stated for the fish oil alone.

Chemicals for hydroperoxide and electron spin resonance (ESR) determination and standards for identification of volatile oxidation products were all obtained from Sigma Aldrich, Steinheim, Germany. All solvents were of HPLC grade from Laboratory-Scan, Dublin, Ireland. Citrem LR 10 Extra (citric acid ester of mono- and diglyceride) without antioxidants added was obtained from Danisco A/S, Grindsted, Denmark. Tween 80 (polyoxyethylene sorbitan monooleate) SigmaUltra was obtained from Sigma Aldrich. Iron(II) sulfate heptahydrate and imidazole were obtained from Merck, Darmstadt, Germany, and sodium acetate trihydrate was obtained from J. T. Baker, Deventer, The Netherlands.

Table 1. Experimental Design

emulsifier	phenolic compounds	phenol concn, mg/kg (mol/kg)	pH	$FeSO_4$	sample name
		Experiment 1			
Citrem	naringenin	100 (3.67×10^{-4})	3	+	Nar3FeCi
Citrem	naringenin	100 (3.67×10^{-4})	3		Nar3 Ci
Citrem	rutin	100 (1.64×10^{-4})	3	+	Rut3FeCi
Citrem	rutin	100 (1.64×10^{-4})	3		Rut3 Ci
Citrem	caffeic acid	100 (5.55×10^{-4})	3	+	Caf3FeCi
Citrem	caffeic acid	100 (5.55×10^{-4})	3		Caf3 Ci
Citrem	control		3	+	Con3FeCi
Citrem	control		3		Con3 Ci
Citrem	naringenin	100 (3.67×10^{-4})	6	+	Nar6FeCi
Citrem	naringenin	100 (3.67×10^{-4})	6		Nar6 Ci
Citrem	rutin	100 (1.64×10^{-4})	6	+	Rut6FeCi
Citrem	rutin	100 (1.64×10^{-4})	6		Rut6 Ci
Citrem	caffeic acid	100 (5.55×10^{-4})	6	+	Caf6FeCi
Citrem	caffeic acid	100 (5.55×10^{-4})	6		Caf6 Ci
Citrem	control		6	+	Con6FeCi
Citrem	control		6		Con6 Ci
		Experiment 2			
Tween80	coumaric acid	100 (6.09×10^{-4})	6	+	Cou6feTw
Tween80	coumaric acid	100 (6.09×10^{-4})	6		Cou6tw
Tween80	caffeic acid	100 (5.55×10^{-4})	6	+	Caf6FeTw
Tween80	caffeic acid	100 (5.55×10^{-4})	6		Caf6Tw
Tween80	control		6	+	Con6FeTw
Tween80	control		6		Con6Tw
Citrem	coumaric acid	100 (6.09×10^{-4})	6	+	Cou6FeCi
Citrem	coumaric acid	100 (6.09×10^{-4})	6		Cou6 Ci
Citrem	caffeic acid	100 (5.55×10^{-4})	6	+	Caf6feCi
Citrem	caffeic acid	100 (5.55×10^{-4})	6		Caf6 Ci
Citrem	control		6	+	Con6FeCi
Citrem	control		6		Con6 Ci

The phenolic compounds (±)-naringenin (≈95%), rutin hydrate (>95%), and caffeic acid (>99%) were all obtained from Sigma Aldrich. *p*-Coumaric acid (≥98%) was obtained from Fluka Chemie, Buchs, Germany.

Preparation of Oil-in-Water Emulsions. The emulsion consisted of 10% oil (MCT/fish oil 1:1, liquid at room temperature), 1% emulsifier, 89% 10 mM acetate−imidazole buffer (pH 3 or 6), and 100 mg/kg phenolic compound. Oil-in-water emulsions were prepared in two steps: (1) pre-emulsification and (2) homogenization. During the pre-emulsification step, the buffer was stirred with an Ultra-Turrax (Janke & Kunkel IKA-Labortechnik, Staufen, Germany) for a few seconds, and thereafter the oil−emulsifier solution was added during 1 min under vigorous mixing followed by 2 min of mixing. The emulsion was then homogenized five times (pressure = 80 and 800 bar) at room temperature using a table homogenizer from GEA Niro Soavi SPA (Parma, Italy).

Experimental Protocol. The phenolic compounds were solubilized in ethanol. Appropriate amounts of phenols (100 ppm) and iron (100 μM) as $FeSO_4$ were added to the emulsion after the homogenization according to the experimental design (**Table 1**). The samples were stored in 100 mL Blue Cap bottles at 20 ± 3 °C without caps in a box covered by a dish towel in the dark. Samples were taken at days 0, 2, 5, and 7 and stored in separate brown glass bottles at −40 °C until analysis of peroxide values and volatiles was performed. Determination of free radicals was performed immediately after sampling without prefreezing. The surface charge of the droplets (zeta potential) and droplet size were determined at day 1 without prefreezing.

Droplet Size. Droplet size was determined by laser diffraction with a Mastersizer2000 (Malvern Instruments Ltd., Worcestershire, U.K.). Approximately 0.25 mL of oil-in-water emulsion was diluted directly in the recirculating water (3000 rpm), reaching an obscuration at 15–18%. The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively. Results are given in surface mean diameter $D_{3,2} = (\Sigma n_i d_i^3/\Sigma n_i d_i^2)$ and volume mean diameter $D_{4,3} = (\Sigma n_i d_i^4/\Sigma n_i d_i^3)$.

Zeta Potential Measurement. The surface charge was determined by measurement of the zeta potential with a ZetaSizer 4 (Malvern Instruments Ltd.) at room temperature. The zeta potential range was set to −100 to 50 mV. The experiment duration was 20 s. Each sample was diluted in 10 mM acetate−imidazole buffer, pH 3 or 6 (about 0.05 mL of the sample was diluted with 25 mL of buffer), before the measurements.

Determination of Free Radicals by Electron Spin Resonance (ESR). ESR measurements were performed using a spin-trapping technique. The spin trap *N-tert*-butyl-α-phenylnitrone (PBN) was added directly to the emulsions to a final concentration of 30 mM. The emulsions were placed in a water bath set at 50 °C immediately after the addition of PBN and heated during 40 min. ESR spectra were recorded using a Bruker ECS 106 X band spectrometer with a 9216 cavity (Bruker, Rheinstetten, Germany). The instrument settings used were as follows: modulation amplitude, 0.508 G; time constant, 164 ms; conversion time, 164 ms; modulation frequency, 100 kHz; microwave power, 20 mW; attenuation, 10 dB; microwave frequency, 9.76 GHz; center field, 3476 G; sweep width, 50 G. The emulsions were placed in a quartz ESR flat cell, and spectra were recorded at ambient temperature. The tendency of radical formation was measured by the growth of the relative signal height of the spin adduct as the signal height was found to give more reproducible results than the signal area.

Determination of Peroxide Value. Lipids were extracted from the emulsions according to a modified Bligh and Dyer method (*17*) using a reduced amount of the chloroform/methanol (1:1 w/w) solvent (*18*). PV was measured by the colorimetric ferric−thiocyanate method (*19, 20*).

Determination of Volatile Compounds. *Headspace Sampling.* The volatile compounds were sampled by the dynamic headspace technique. Four grams of emulsion, 30 mg of 4-methyl-1-pentanol (internal standard), and 1 mL of synperonic (antifoam) were purged with nitrogen at 150 mL/min in 30 min at 45 °C. Antifoam was used to prevent the formation of foam, which would lead to condensation of water and contamination from the sample on the Tenax GR material. The volatile compounds were collected on traps (Perkin-Elmer, Hartford, CT) packed with 225 mg of Tenax GR (Chrompack, Bergen op Zoom, The Netherlands).

Thermal Desorption and GC-MS. An ATD-400 automatic thermal desorber with a Tenax GR-packed cold-trap (Perkin-Elmer) was used for thermally desorbing the collected volatiles. Helium was used as carrier gas with a flow of 1.3 mL/min. The transfer line of the ATD was connected to a 5890 IIA gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a DB 1701 column (30 m × 0.25 mm × 1.0 μM, J&W Scientific, Folsom, CA) coupled to a HP 5972A mass selective detector. The temperature program used was as follows: 45 °C for 5 min, raised from 45 to 55 at 1.5 °C/min, raised from 55 to 90 at 2.5 °C/min, raised from 90 to 220 °C at 12 °C/min, and finally held at 220 °C for 4 min. The GC-MS transfer line temperature was kept at 280 °C. The ionization energy of the mass spectrometer was set at 70 eV in the EI mode, and the detector operated with a mass range between 30 and 250 with a scan rate at 3.35 scans/s.

Identification and Quantification of Secondary Volatile Oxidation Compounds. Compounds were identified by MS library searches and by comparing retention time and spectra with MS runs of external standards. On the basis of knowledge from previous studies (*21–24*) 13 volatiles were selected for further quantification through calibration curves. The selected volatiles were 1-penten-3-one, pentanal, 1-penten-3-ol, 2(t)-pentenal, hexanal, 2(t)-hexenal, heptanal, 2(t)-heptenal, octanal, 2,4(t,t)-heptadienal, nonanal, 2(t)-nonenal, and 2(t)-decenal.

Quantification of the compounds released from the emulsion systems was performed by selected ion monitoring. The target ion represents a specific MS fragmentation ion of each compound. The target ions were verified on the basis of two or three qualifier ions and the chromatographic retention time. The calibration curves were determined by applying 1.0 μL of the solution of volatiles at five concentration levels directly to Tenax tubes. Determinations were made in triplicate with relative standard derivations ranging from 0.1 to 4% and with correlation coefficients for all compounds between 0.993 and 0.998.

Adsorption of Antioxidants at Emulsion Droplet Surfaces. A model emulsion was prepared by emulsifying 10% MCT oil in deionized water using 0.3% emulsifier dissolved in the oil phase using a small-scale home-built high pressure homogenizer (*25*). The pressure

was adjusted to obtain about 2 μm sized emulsion droplets. The particle size was evaluated using laser light diffraction (Coulter LS-30, Beckman Coulter, High Wycombe, U.K.). The specific surface area was estimated from $D_{3,2}$.

Polyphenol stock solutions were prepared by dissolving the phenolic compounds in methanol (1% solution) and thereafter diluted with 0.02 M acetate/lactic acid/imidazole buffer (pH 3, 4, 5, and 6). Normally the stock solution was 100 mg/kg. The stability of the solution was checked after 24 h at 6 °C. If visible signs of polyphenol precipitation were observed (turbidity or crystal growth at glass surfaces), a more diluted solution was prepared (dilution 1:1 with buffer). This protocol was used to estimate the solubility of the polyphenol. The stock polyphenol solution was added to 5 mL of emulsion in amounts corresponding to either 2 or 1 mg of phenolic compound/m^2 counted on the emulsion surface area. The sample volume was adjusted with buffer to 11 mL.

The samples were equilibrated for 24 h at 6 °C, and after equilibration, the samples were centrifuged for removal of emulsion droplets (4000g, 10 min). The centrifugation time and the speed were adjusted to avoid coalescence between the oil droplets but still sufficient to obtain a clear supernatant. The remaining phenolic compound in the supernatant was analyzed using HPLC. The HPLC system consisted of a Shimadzu (LC 10ADVP) liquid chromatograph system, a vacuum degasser (DGU 14-A), a solvent delivery module (FCV-10ALVP), an autoinjector (SIL-10ADVP), a column oven (CTO-10ASVP), and diode array detector (SPD-M10AVP). The column used was a 3.5 μm Kromasil reversed phase column 150 mm × 4 mm protected by a Kromasil C 18 10 mm precolumn. The flow rate was 0.8 mL/min, and the injection volume was 20 μL. The mobile phase was a binary solvent system consisting of methanol and a 1% acetic acid/water mixture (from 40 to 90% methanol). UV spectra were detected at 280 nm. Quantification was carried out by comparing the peak heights of the samples with reference standards. The signal height versus signal area was evaluated statistically using reference solutions at known concentrations. On the basis of a slightly better precision and accuracy we decided to use signal height. Analyses were made in duplicate. The adsorbed amount was obtained by

$$\Gamma = \frac{c_{\text{initial}} - c_{\text{equilibrium}}}{s_{\text{emulsion}}}$$

where c_{initial} and $c_{\text{equilibrium}}$ refer to the initial and final polyphenol concentrations (mg/mL), s_{emulsion} is the emulsion droplet area (m^2/mL), and Γ is the adsorbed amount of polyphenol (mg/m^2).

Evaluation of Antioxidant–Iron Interactions by Spectroscopy. The different phenolic compounds (caffeic acid, coumaric acid, naringenin, and rutin) were solubilized in ethanol and added to an acetate−immidazol buffer (10 mM sodium acetate and 10 mM immidazol, pH 3 and 6). The concentration of phenolic compound in the buffer was 100 mg/kg. For all phenolic compounds, solutions with only the different phenolic compounds and buffer and one with the different phenolic compounds, buffer, and iron were measured. Buffer at the respective pH values (pH 3 or 6) without iron was used as reference. Each sample was measured using a UV spectrophotometer (UV 160, Shimadzu, Columbia, MD) and a spectrum was obtained.

Evaluation of Phenol–Iron Interactions by Cryogenic Transmission Electron Microscopy (Cryo-TEM). Cryo-TEM is a special TEM technique that allows the investigation of samples without traditional sectioning. To investigate the interaction between iron and caffeic/coumaric acid at pH 6, caffeic acid and coumaric acid were dissolved in pure water and the pH of the solution was adjusted. Precipitation was initiated by adding an amount of each solution to an Fe^{2+}-containing aqueous solution (pH 6.0) to obtain the desired concentration (100–300 mg/kg phenolic compound and 100 μmol/L Fe^{2+}). The tube was rapidly mixed and left to stand at room temperature for 24 h, after which a droplet was placed on a lacy carbon film supported by a copper grid and gently blotted with filter paper to obtain a thin liquid film. The grid was quenched in liquid ethane (−180 °C) and transferred to liquid nitrogen (−196 °C). The samples were transferred to the TEM (Philips CM120 BioTWIN Cryo) equipped with an energy filter imaging system (Gatan GIF 100) and an Oxford CT 3500 cryoholder and transfer system. The acceleration voltage was 120 kV and the working temperature −180 °C.

Table 2. Droplet Size of the Emulsion with the Different Emulsifiers Applied at pH 3 and 6[a]

emulsifier	pH	$D_{3,2}$, μm	$D_{4,3}$, μm
Citrem	3	0.320 ± 0.006	0.656 ± 0.044
Tween80	6	0.142 ± 0.006	0.518 ± 0.150
Citrem	6	0.142 ± 0.006	0.532 ± 0.098

[a] $D_{3,2}$ (μm) = surface mean diameter; $D_{4,3}$ (μm) = volume mean diameter.

Statistical Analysis. The data obtained by PV, ESR, and zeta potential were analyzed by one-way or two-way analysis of variance (ANOVA), and individual samples were compared on a 0.05 level of significance by the Bonferroni multiple comparison. This test allows comparsion of selected pairs of columns (variables) for significant differences (*26*). The volatile oxidation product data were subjected to an ANOVA partial least-squares analysis using Unscrambler version 9.0 (Camo, Oslo, Norway). This analysis is a partial least-squares regression, but instead of using measured data as *X*- and *Y*-data, the variables describing the design are used as *X*-data and the measured data are used as *Y*-data. The design variables used as *X*-variables were emulsifier type (Citrem, Tween), pH (3 and 6), and phenolic compounds (naringenin, rutin, caffeic acid, coumaric acid, and control). The measured values were volatile oxidation products. All variables were weighted (1/standard deviation), and the models were cross-validated by using the different codes as segments. By using the jack-knifing facility in the Unscrambler software, it was possible to assess whether regression coefficients for the different design variables were significantly positive or negative ($p < 0.05$) for each of the measured variables.

RESULTS

Physical Properties of the Emulsions. Droplet size was measured for the three different emulsions, Citrem pH 3, Citrem pH 6, and Tween pH 6, before addition of the different phenolic compounds and iron. The measured droplet sizes of the three different emulsions indicated that the droplet sizes were similar at pH 6 irrespective of the type of emulsifier (**Table 2**). However, at pH 3 $D_{3,2}$ was significantly larger compared to the same emulsion at pH 6, whereas the difference between samples was not significant for $D_{4,3}$ (**Table 2**).

The droplet charge of the Citrem-stabilized emulsions was strongly negative as Citrem is an anionic emulsifier, and the charge was significantly affected by pH (**Figure 2**). At pH 6 the Citrem-stabilized emulsion had a charge of around −45 to −35 mV and at pH 3 a charge of around −20 mV. At pH 3 there were no significant differences between the droplet charges in the emulsions when different phenolic compounds were applied. However, in emulsion without iron added the droplet charge with any of the phenolic compounds was significantly more negative compared to the emulsion without phenolics (control). This indicated that the phenolic compounds interacted with Citrem. In Citrem-stabilized emulsion at pH 6 without iron, the emulsion with rutin added had significantly more negatively charged droplets compared to emulsions with other phenolics at the same pH. Surprisingly, addition of iron generally resulted in more negatively charged droplets in Citrem-stabilized emulsions at pH 6 (**Figure 2**), and for caffeic acid, coumaric acid, and naringenin the changes in droplet charge were significant. The most negatively charged emulsion droplets with iron added were obtained with caffeic acid ≫ rutin > naringenin > coumaric acid > control. These data indicated that iron interacted with the phenolic compounds. Emulsions stabilized by Tween had a charge of around −2 mV as Tween is a nonionic emulsifier. In contrast to the Citrem-stabilized emul-

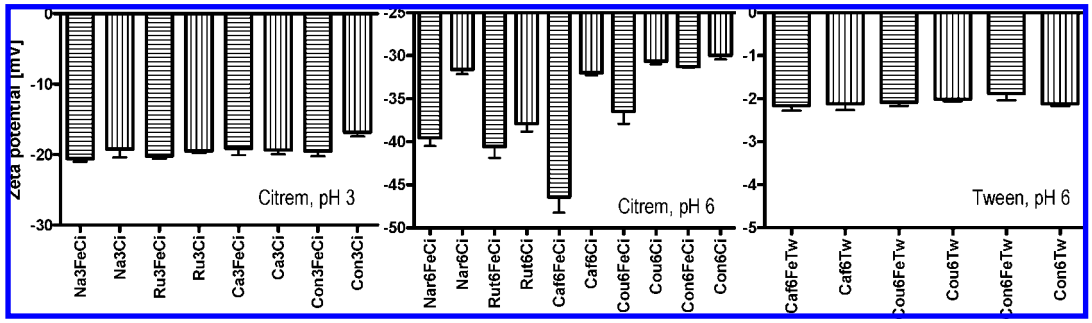

Figure 2. Zeta potential measured in the different emulsions stabilized with Citrem (pH 3 and 6) and Tween (pH 6). Sample names refer to **Table 1**. Bars indicate the standard deviation of three measurements.

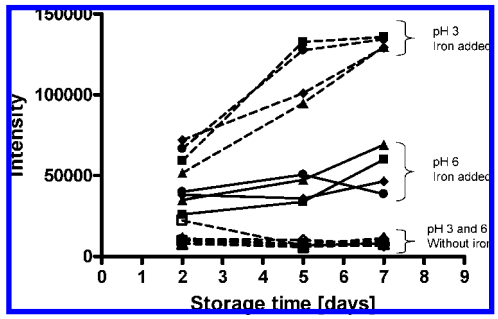

Figure 3. Relative signal height of electron spin resonance spectra for the different emulsions stabilized by Citrem at pH 3 (dotted lines) and at pH 6 (straight lines): (●) naringenin + Fe; (○) naringenin; (■) rutin + Fe; (□) rutin; (▲) caffeic + Fe; (△) caffeic; (◆) control + Fe; (◇) control. Only one measurement was performed.

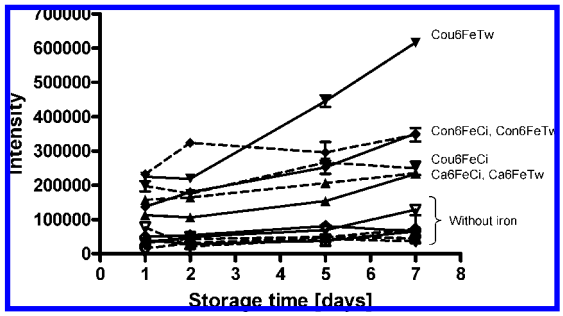

Figure 4. Relative signal height of electron spin resonance spectra for the different emulsions at pH 6 stabilized with Tween (straight line) and Citrem (dotted line), respectively: (▼) coumaric acid + Fe; (▽) coumaric acid; (▲) caffeic acid + Fe; (△) caffeic acid; (◆) control + Fe; (◇) control. Sample names refer to **Table 1**. The bars indicate the standard deviations of three measurements ($n = 3$).

sions at pH 6, the charge of the droplets in the Tween-stabilized emulsions did not change significantly, irrespective of the presence of phenols and iron.

Determination of Tendency of Radical Formation by ESR. ESR measurements showed that addition of iron had a prooxidative effect. Samples containing iron had not only a higher formation of spin adducts but also increased spin adducts during storage (**Figures 3** and **4**). Furthermore, samples with pH 3 showed a higher oxidation level (radical formation) than those at pH 6 (**Figure 3**). With Citrem as emulsifier, no antioxidative effect was observed by addition of the phenols compared to the control samples (**Figure 3**). On the contrary, a slight prooxidative effect was observed for the samples containing iron and naringenin or rutin after 5 days of storage at pH 3 (**Figure 3**) and for samples containing iron with rutin or caffeic acid at day 7 and pH 6 (**Figure 3**). In Tween-stabilized emulsions, coumaric acid together with iron had a prooxidative effect and caffeic acid had an antioxidative effect compared to the sample without phenols. However, without iron both phenols showed a slight antioxidative effect in Tween-stabilized emulsions. In Citrem-stabilized emulsions at pH 6 and in the presence of iron both coumaric acid and caffeic acid reduced the oxidation levels compared to the emulsion without any phenols added (**Figure 4**). No significant effect of the two phenols was observed without iron added. Thus, different effects of the phenols were observed in emulsions made with Tween and iron compared to those with Citrem and iron. The emulsifier did not influence the oxidation level significantly in the samples without phenols added. However, emulsion with coumaric acid and iron had a significantly higher oxidation level in Tween-stabilized emulsion than in Citrem-stabilized emulsion.

Peroxide Value (PV). PV increased significantly during the storage period (1 week), and the concentrations were between 1.9 and 68.3 mequiv of peroxides/kg of oil, between 1.4 and 15.0 mequiv of peroxides/kg of oil, and between 2.0 and 21.6 mequiv of peroxides/kg of oil in the emulsions stabilized with Citrem at pH 3, Citrem at pH 6, and Tween at pH 6, respectively (**Figures 5** and **6**). In emulsions stabilized with Citrem at pH 3 without the presence of iron, PV increased only slightly (**Figure 5A**). In the presence of iron in the Citrem-stabilized emulsions, caffeic acid reduced PV the first 2 days, but thereafter it promoted formation of peroxides. Likewise, naringenin and rutin increased the level of peroxides compared to the control. The highest PV was observed at day 7 in emulsion with caffeic acid (~70 mequiv of peroxides/kg of oil) > rutin > naringenin ≥ control. Interestingly, with the same emulsifier at pH 6 the exact opposite ranking of the phenolic compounds was observed, and the ranking was similar in the emulsions with and without iron added. Thus, at this pH caffeic acid and rutin inhibited the development of PV during the entire storage period (**Figure 5B**). Generally, the use of a nonionic emulsifier, Tween, resulted in a significantly higher concentration of peroxides compared to the use of an anionic emulsifier, Citrem. However, the effect of caffeic acid on the development of peroxides was unaffected by the emulsifier used (**Figure 6**; **Table 3**). Thus, caffeic acid in emulsions stabilized by Tween or Citrem at pH 6 significantly reduced PV. For emulsion with coumaric acid, there was a slight influence of the emulsifier. In Citrem-stabilized emulsion, coumaric acid slightly decreased PV, whereas in Tween-stabilized emulsion coumaric acid slightly increased PV or had no effect (**Figure 6**; **Table 3**). Overall, the presence of iron in the emulsions had a significantly negative effect on the oxidative stability determined from the concentration of peroxides.

Volatiles. The concentrations of the following volatile secondary oxidation products were measured during storage:

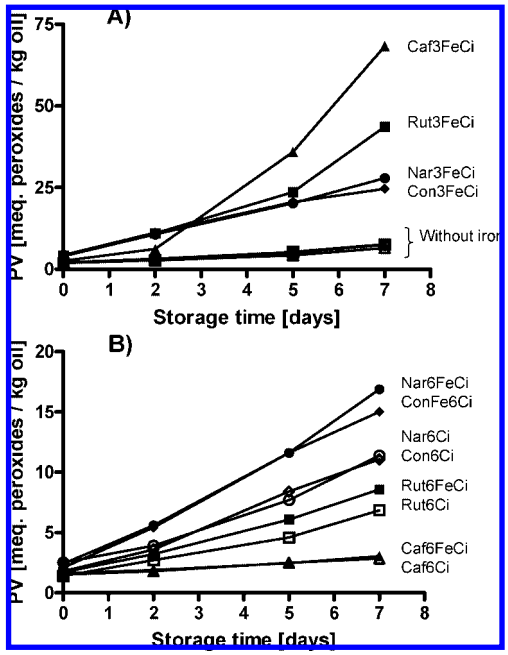

Figure 5. PV concentration during storage measured in the different oil-in-water emulsions with different phenolic compounds with or without iron added: (●) naringenin + Fe; (○) naringenin; (■) rutin+ Fe; (□) rutin; (▲) caffeic + Fe; (△) caffeic; (◆) control + Fe; (◇) control. The emulsions were stabilized by Citrem at pH 3 (**A**) or at pH 6 (**B**). The bars indicate the standard deviations of three measurements ($n = 3$). Sample names in the graph refer to **Table 1**.

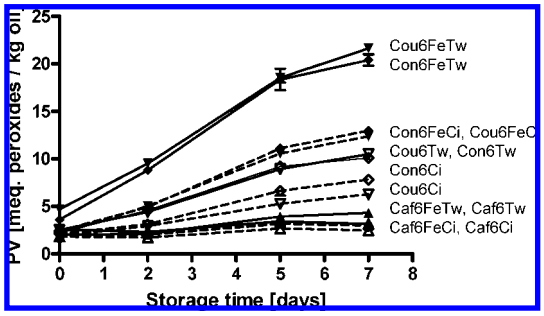

Figure 6. PV during storage measured in oil-in-water emulsions with different phenolic compounds (caffeic acid, coumaric acid, and control) with or without iron added stabilized by Citrem (dotted line) or Tween (solid line): (▼) coumaric acid + Fe; (▽) coumaric acid; (▲) caffeic acid + Fe; (△) caffeic acid; (◆) control + Fe; (◇) control. The bars indicate the standard deviations of three measurements ($n = 3$). Sample names in the graph refer to **Table 1**.

1-penten-3-one, pentanal, 1-penten-3-ol, 2-pentenal, hexanal, 2-hexenal, heptanal, 2-heptenal, octanal, 2,4-heptadienal, nonanal, 2-nonenal, and 2-decenal. Nonanal and 2-decenal could not be identified with certainty and may be contaminated by other volatile compounds. Many of the detected volatiles have previously been identified in boiled fish (*23*) and in fish oil (*27*) and have been shown to correlate with the degree of oxidation in fish oil enriched emulsions (*27, 28*). As examples, pentanal and hexanal originate from n-6 fatty acids (*1*), and 1-penten-3-one, 2-hexenal, and 2,4-heptadienal all originate from marine long-chain n-3 PUFA (*29*).

Similar to PV and formation of radicals (ESR), the concentration of volatiles was higher in emulsions with iron than in emulsions without iron (compare panels **A** and **B** and panels **C** and **D** of **Figure 7**). The data obtained in Citrem-stabilized emulsions with iron at pH 3 were very clear. Thus, the

Table 3. Overview of the Significant Effect of the Different Phenolic Compounds Applied in Experiments 1 and 2[a]

	phenolic compound							
	NarFe	Nar	RutFe	Rut	CafFe	Caf	CouFe	Cou
				Citrem, pH 3				
PV-0	no	no	no	no	anti	no	–	–
PV-2	no	no	no	no	anti	no	–	–
PV-5	no	no	pro	no	pro	anti	–	–
PV-7	pro	no	pro	no	pro	anti	–	–
				Citrem, pH 6				
PV-0	no	pro	anti	anti	anti	no	no	no
PV-2	no	no	anti	anti	anti	anti	no	no
PV-5	no	anti	anti	anti	anti	anti	anti	anti
PV-7	pro	no	anti	anti	anti	anti	anti	anti
ESR-1	–	–	–	–	anti	no	anti	pro
ESR-2	–	–	–	–	anti	no	anti	no
ESR-5	–	–	–	–	anti	no	no	no
ESR-7	–	–	–	–	anti	no	anti	no
				Tween, pH 6				
PV-0	–	–	–	–	anti	no	no	no
PV-2	–	–	–	–	anti	anti	no	no
PV-5	–	–	–	–	anti	anti	no	no
PV-7	–	–	–	–	anti	no	pro	no
ESR-1	–	–	–	–	anti	no	pro	no
ESR-2	–	–	–	–	anti	anti	pro	no
ESR-5	–	–	–	–	anti	anti	pro	anti
ESR-7	–	–	–	–	anti	anti	pro	pro

[a] Antioxidative ("anti") indicates that the particular emulsion had a significant lower level, and prooxidative ("pro") indicates that the particular emulsion had a significantly higher level of PV and radicals (ESR) compared to the same emulsion without any phenolic compound added. A dash indicates that it was not measured. "No" indicates that no significant effect was observed. The significant effects were evaluated by the Bonferroni analysis on the quantitative data. Abbreviations: NarFe, naringenin + Fe; Nar, naringenin; RutFe, rutin + Fe; Rut, rutin; CafFe, caffeic acid + Fe; Caf, caffeic acid; CouFe, coumaric acid + Fe; Cou: coumaric acid.

concentration of all the evaluated volatiles was observed to be highest in emulsions with caffeic acid followed by rutin > naringenin ≥ control, as also shown for the sum of volatile concentrations in **Figure 7A**. Moreover, regression coefficients from an ANOVA partial least squares regression (APLSR model) on the individual volatiles confirmed this interpretation of the raw data (data not shown). In contrast, the data from the Citrem emulsions at pH 3 without iron were more complex. Thus, there were almost no differences between the concentrations of individual volatiles in emulsions with different phenolic compounds. The multivariate analysis including the regression coefficients indicated that rutin and naringenin promoted the formation of nonanal and heptanal, whereas concentrations of the other volatiles evaluated were highest in emulsions with caffeic acid (data not shown). Again, the sum of all volatiles was higher in the emulsions with caffeic acid than in the other emulsions (**Figure 7**). In Citrem- or Tween-stabilized emulsions at pH 6, the development of volatiles was much slower compared to that in Citrem-stabilized emulsions at pH 3 (**Figure 7**). In emulsions at this pH without iron added, the formation of volatiles was affected by both the different phenolic compounds and the type of emulsifier applied. However, only the emulsifier type and caffeic acid had significant effects on the formation of individual volatiles (data not shown). The concentrations of different volatiles were significantly higher in emulsions stabilized by Tween compared to Citrem as also observed for the sum of all measured volatiles (**Figure 7D**). Moreover, in the emulsions with Tween, caffeic acid was prooxidative and the other phenols had no significant antioxidative effects. Interestingly, in emulsions with Citrem without iron, the sum of all volatiles suggested a small antioxidative

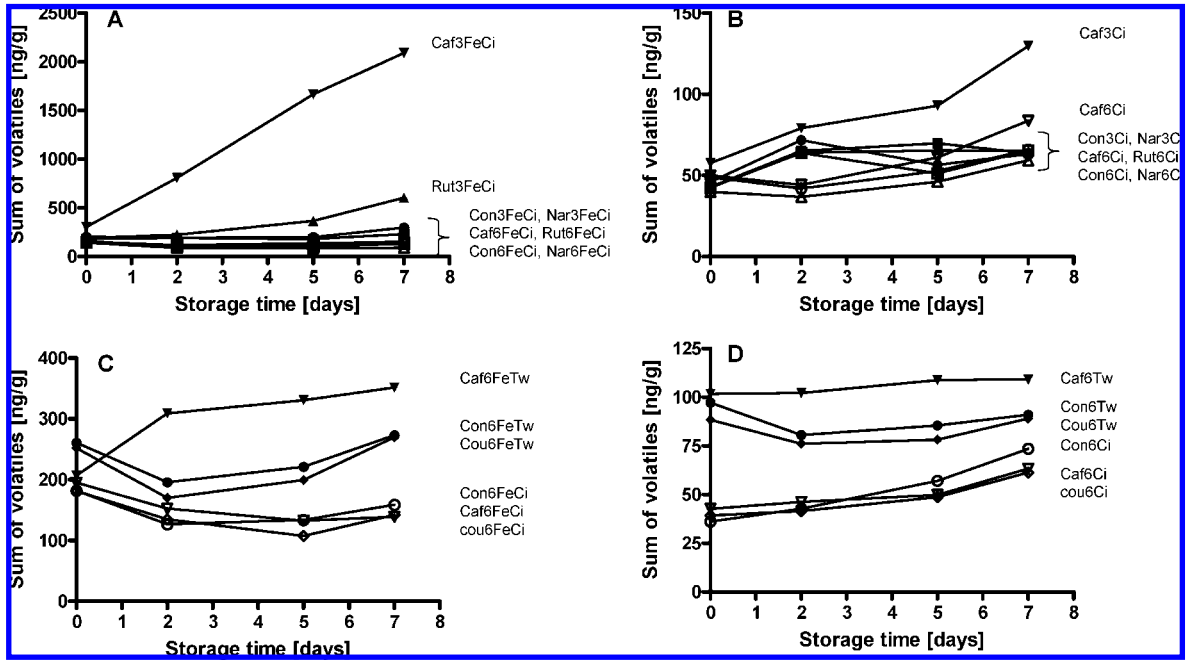

Figure 7. Sum of the measured volatiles (ng/g; 1-penten-3-one, pentanal, 1-penten-3-ol, 2-pentenal, hexanal, 2-hexenal, heptanal, 2-heptenal, octanal, 2,4-heptadienal, nonanal, 2-nonenal, and 2-decenal): (**A**) phenolic compounds (naringenin, rutin and caffeic acid) at pH 3 and 6, Citrem as emulsifier with iron added; (**B**) phenolic coumpounds (naringenin, rutin, and caffeic acid) at pH 3 and 6, Citrem as emulsifier without iron added; (**C**) phenolic compounds (coumaric and caffeic acid) at pH 6, Tween and Citrem as emulsifier with iron added; (**D**) phenolic compounds (coumaric and caffeic acid) at pH 6, Tween and Citrem as emulsifier without iron added. Sample names refer to **Table 1**.

Table 4. Effect of the Different Phenolic Compounds and Emulsifier on Formation of Volatiles[a]

	emulsifier/phenol compound					
	Citrem	Tween	Caf	Cou	Nar	Rut
pH 3 without iron	–	–	pro	–	no	no
pH 3 with iron	–	–	pro	–	anti	anti
pH 6 without iron	anti	pro	pro	no	no	no
pH 6 with iron	no	no	no	no	no	no

[a] Antioxidative ("anti") indicates that the particular emulsion had a significant lower level, and prooxidative ("pro") indicates that the particular emulsion had a significantly higher level of volatile oxidation products as evaluated from regression coefficients from the APLSR analysis. "No" indicates no significant effects of the current emulsifier or phenolic compounds, and a dash indicates that the current emulsifier or phenolic compound was not measured. Abbreviations: Caf, caffeic acid; Con, control; Cou, coumaric acid; Nar, naringenin; Rut, rutin.

effect of caffeic acid and coumaric acid. In emulsions with iron added, there were no significant effects of the applied emulsifier or phenolic compounds on the individual volatiles (data not shown). Here, the effect of iron addition was highly prooxidative, which may have masked possible effects of the emulsifier and the different phenolic compounds on the oxidation in emulsions during storage. However, the sum of all measured volatiles suggested that caffeic acid had a prooxidative effect in Tween emulsions (**Figure 7C**) as was also the case in emulsions without iron, but in the Citrem emulsions no clear effect of the phenolic compounds could be observed.

The significant effects of the different phenolic compounds on development of PV and formation of radicals are summarized in **Table 3**. Moreover, the general effects of emulsifier and phenolic compounds on the formation of volatile oxidation products are summarized in **Table 4**.

Phenol−Emulsifier Interactions. The antioxidative function of the phenols in this study may be related to their aqueous solubility as well as their ability to adsorb at the surface of the emulsion droplets. To check this hypothesis the adsorption of phenols at emulsion droplets was investigated. The aqueous solubility of narigenin (60 mg/kg) and rutin (80 mg/kg) was limited, whereas it was higher for caffeic (320 mg/kg) and coumaric acid (640 mg/kg). The solubility is a factor that needs to be considered when the functionality of the substances is discussed.

The results of the adsorption measurements are displayed in **Table 5**. A clear adsorption at the interface was observed only for naringenin, from around 0.2 to 0.6 mg/m^2. A typical emulsifier layer is in the range of 2 mg/m^2. Hence, this range of adsorption corresponds to an interfacial layer that contains about 20–25% of naringenin. For the other phenols only insignificant adsorption was detected.

Phenol–Iron Interaction. Interactions between the different phenolic compounds and iron were determined on the basis of UV spectrophotometry measurements and by observations of formed nanoparticles by cryo-TEM for caffeic and coumaric acid. A spectrum (200–800 nm) was obtained for each phenolic compound dispersed in buffer with and without iron added. When iron was added to the buffer without phenolic compound, the spectrum did not change (data not shown). This indicated that the applied buffer (pH 3 or 6) and addition of iron did not interact with each other. At pH 6 the UV absorption changed when iron was added to the buffer containing different phenolic compounds: rutin, naringenin, caffeic acid, and coumaric acid (**Figure 8**, rutin). At this pH value, rutin and caffeic acid absorbed light in the visible area, which indicated formation of a catechol−iron complex. Furthermore, caffeic acid and coumaric acid were shown to form nanoparticles in the presence of iron as exemplified in **Figure 9** for caffeic acid. Such particles were observed at a concentration of 100–300 mg/kg of caffeic acid or coumaric acid in the presence of 100 μmol/L Fe^{2+} at pH 6. The hydrodynamic radius (the radius of a sphere with a similar diffusion coefficient) of the particles was approximately 20 nm, in the case of caffeic acid, and 100–200 nm, in the case

Table 5. Adsorption (mg/m^2) of Phenolic Compounds at the Emulsion Droplet Surface[a]

		naringenin[b]		rutin[b]		caffeic acid[c]		coumaric acid[c]	
	pH	1 mg/m^2	2 mg/m^2	1 mg/m^2	2 mg/m^2	1 mg/m^2	2 mg/m^2	1 mg/m^2	2 mg/m^2
Citrem	6	0.18	0.45	0.04	0.05	−0.01[d]	0.00	−0.01	−0.07
	5	0.19	0.42	0.04	0.09	−0.07	−0.05	−0.04	−0.04
	4	0.20	0.38	−0.02	0.01	0.03	0.03	−0.02	0.03
	3	0.18	0.40	−0.01	−0.06	−0.05	0.06	−0.06	0.07
Tween	6	0.30	0.49	0.04	−0.14	−0.05	−0.10	0.01	−0.04
	5	0.29	0.53	0.01	0.02	−0.03	−0.07	−0.03	−0.07
	4	0.29	0.57	0.03	0.01	−0.02	−0.12	−0.02	−0.08
	3	0.30	0.57	−0.01	0.01	0.01	−0.04	−0.02	−0.04

[a] The concentration of polyphenol added was adjusted to the present interfacial area to correspond to maximal adsorption of either 1 or 2 mg/m^2. [b] Average of four measurements. [c] Average of two measurements. [d] Negative values are possibly caused by a more rapid precipitation of solid polyphenol in the reference than in the emulsion sample.

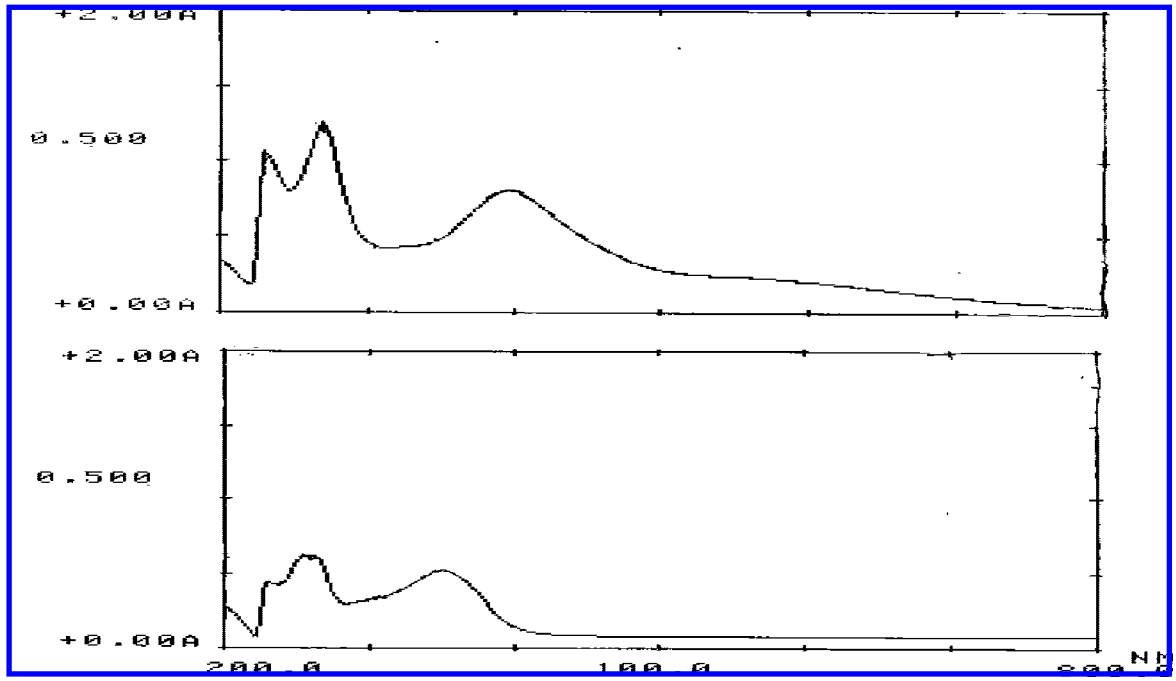

Figure 8. UV spectrum for rutin soublized in pH buffer: (**A**) rutin with iron added; (**B**) rutin without iron added.

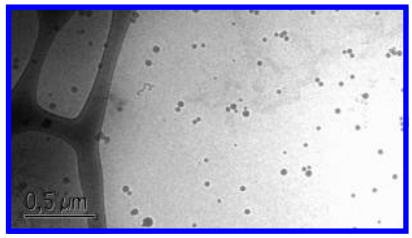

Figure 9. Cryo-TEM micrograph of Fe−caffeic acid particles. The concentrations of Fe^{2+} and caffeic acid were 100 μmol/L and 100 mg/kg, respectively.

of coumaric acid. The particle sizes were confirmed with dynamic light scattering.

Naringenin was the only phenolic compound that changed UV absorption pattern at pH 3 when iron was added as measured by spectroscopy (data not shown). This indicates that only naringenin and iron interacted regardless of pH (pH 3 or 6). One may speculate that the increase in UV−vis absorbance in the presence of phenolics and iron could be due to polymerization of oxidized phenolics. However, for the concentrations investigated, oxidation of phenolics will result in a too low concentration of oxidized phenols for significant polymerization.

DISCUSSION

The structures of both the phenolic compounds and the emulsifier as well as the pH and iron influenced the oxidative stability of the emulsions. Briefly, the main findings were as follows: (1) Lipid oxidation increased when pH was decreased from pH 6 to pH 3. (2) Iron addition significantly increased lipid oxidation. (3) Lipid oxidation was slightly higher in Tween-stabilized emulsions compared with Citrem-stabilized emulsions. (4) The effect of the different phenolic compounds was dependent on the pH of the emulsions and iron addition as well as the emulsifier type. (5) The same phenolic compound had different effects on the different parameters measured; for example, caffeic acid reduced the formation of peroxides but promoted the formation of volatiles.

Physical Properties and the Effect on Lipid Oxidation. The emulsifier did not influence the size of the droplets, but the pH did. At pH 3 the diameter of the droplet was significantly larger compared to the measured value at pH 6. According to the literature, initiation of lipid oxidation takes place at the interface between oil and water (*1, 2*), where metal ions react with hydroperoxides. Thus, lipid oxidation may be expected to be more pronounced in emulsions with a larger total surface area compared to emulsions with a smaller total surface area.

However, the literature on this subject is conflicting (*30–33*), and it seems that lipid oxidation is influenced not only by the droplet size but also by other factors such as the thickness and composition of the interface (*2, 33, 34*). The same emulsifier resulted in more oxidation at pH 3 than at pH 6. Emulsions at pH 3 had the smallest total surface area. Thereby, the results confirmed that factors other than droplet size have an impact on lipid oxidation. Thus, the results indicated that pH, especially in the presence of iron, had a greater impact on lipid oxidation than size of the droplets. The increased oxidation at lower pH in the presence of iron is most likely due to the increased solubility of iron at low pH.

Location of iron in the emulsions is influenced by the solubility of iron and the charge of the droplets. Generally, iron ions are attracted to an interface with a negative charge and repelled by a positive charged interface due to the positive charge of iron, and therefore the charge of the emulsifier has been suggested to play an important role in the oxidation rate (*2*). However, in the present study Citrem emulsions oxidized more slowly than Tween emulsions despite the fact that the oil droplets in the Citrem emulsions had a more negative droplet charge than the Tween emulsions. This finding is in accordance with recent data from our laboratory (unpublished data). The ability of Citrem to reduce oxidation is most likely due to its metal chelating properties, which is a well-known property of citric acid esters. Taken together, these data showed that in the presence of iron the metal chelating properties of an emulsifier may be more important than its charge.

Interactions between Iron, Emulsifiers, and Phenolic Compounds and Effect on Lipid Oxidation. At pH 6 the interface in the Citrem emulsions was more negatively charged compared to pH 3. Surprisingly, at pH 6 the charge became more negative when the positively charged iron was present in emulsions with different phenolic compounds compared to the same emulsions without iron. These results might indicate that the phenols and iron at pH 6 interacted near the interface, although the adsorption data in **Table 5** suggested that only a minor part of the phenolic compound adsorbed at the interface. These apparently contradicting results may partly be caused by adsorption of the phenolic compounds at a level below the detection limit in the adsorption experiments (about 0.15 mg/m^2) that still may alter the surface charge. Furthermore, the adsorption experiment used a lower emulsifier concentration (0.3%) than the storage experiment (1%), and therefore another possibility is that the adsorption experiment slightly underestimated the actual adsorption in the storage experiment.

UV–vis spectra of iron and the individual phenolic compounds showed that when iron was added to the phenolics, the absorbance pattern changed for all of the compounds at pH 6 and for naringenin also at pH 3. This finding indicates an interaction between iron and the phenolic compounds. It has been suggested that a change in the absorbance at 587 and 680 nm for complex-bound Fe^{3+} corresponds to iron−gallyl and iron−catechol complexes (blue- and green-colored), respectively (*35*). Thus, the finding that rutin and caffeic acid changed absorbance in the visible region at pH 6, when iron was added, might indicate formation of a catechol−iron complex. However, naringenin also contains a catechol group, but the absorbance pattern changed only in the ultraviolet region. This could suggest either that Fe^{2+} was complex bound or that a group other than the catechol group in naringenin participated in the complex with iron.

For Citrem-stabilized emulsions results for the zeta potential measurements and spectrophotometric measurements on the different phenols were in agreement. Hence, from the spectrophotometric measurements an interaction with iron for all phenols at pH 6 and an additional interaction for naringenin at pH 3 were observed simultaneously with changes in the zeta potential in emulsions with those phenols under the same pH condition. However, changes in zeta potential in Tween-stabilized emulsions were not observed when iron was present. This might indicate that this emulsifier influenced interactions between the phenolic compounds and iron or that the phenols and iron were not located near the droplet interface. This difference between the observations for Tween and Citrem could be due to the very different structures of the two emulsifiers. Thus, Tween is a bulkier molecule than Citrem, and it is possible that this bulky structure prevented the phenolic compounds from interacting with iron. Moreover, when iron is attached to Citrem as an emulsifier (and only to a lesser degree to Tween), it is most likely due to a ligand exchange reaction in the iron−phenol complex, where Citrem coordinates directly to iron and partly replaces one of the phenol ligands.

Complexation between polyphenols and iron may also result in changed solubility and eventual precipitation. Rutin and naringenin already from the beginning have poor solubility. The phenolic acids, caffeic and coumaric, both have high solubility, but both of them precipitated in the presence of iron ions as detected by the formation of nanoparticles in the presence of iron (**Figure 9**).

Paiva-Martins and Gordon suggested that a negative complex between ferric iron and phenolic compounds containing two hydroxygroups [$Ph(OH)_2$] is formed at pH 5.5 (*36*), which is close to the pH where the change in the UV−vis absorption was observed for all phenolic compounds in the presence of iron:

$$[Fe(H_2O)_4(OH)_2]^+ + Ph(OH)_2 \rightarrow [Fe(H_2O)_2(OH)_2Ph(O)_2]^- + 2HO_3^+$$

Thus, the observed increase in negative charge (zeta potential) in the Citrem-stabilized emulsion at pH 6 in the presence of polyphenols most likely is due to the formation of such a complex. Moreover, we suggest that this complex also formed the observed nanoparticles that are adhering at the oil–water interface.

One may expect that complex-bound iron is unable to react with the hydroperoxides or that precipitation of iron by phenolic compounds may lead to reduced oxidation. However, oxidation did occur at pH 6 in the presence of the phenolics and iron to a higher extent than when iron was not present. This could indicate that the complexation of the phenolic compounds could also prevent them from acting as free radical scavengers or that free iron was still present as will be further discussed later.

Mancuso et al. (*12*) hypothesized that under certain circumstances the decrease in water solubility for iron ions at increasing pH may result in a precipitation of iron on the lipid droplets surface and that this may lead to increased oxidation. Our findings that precipitation of the iron−polyphenol complex at the interface did not seem to reduce oxidation to the same level as when iron was not present fits to some extent with this hypothesis.

Anti and Prooxidative Mechanisms of Phenolic Compounds. The hydroxyl groups are the reactive part of the phenolic molecule as they are able to donate H-atoms to free radicals and as they are also responsible for the metal chelating properties of phenolic compounds. Both rutin and caffeic acid are compounds that contain an *o*-diphenol group in their

molecular structure. *o*-Diphenol groups are able to chelate metal ions such as iron.

In Citrem emulsions at pH 3, iron ions are expected to be localized at the negatively charged droplet surface. At this pH caffeic acid strongly promoted oxidation, whereas naringenin and rutin promoted peroxide formation at the end of the storage period, but generally reduced formation of volatiles. The spectrophotometric results showed no interaction between the phenolic compound and iron at pH 3 except for naringenin. Thus, iron is expected to exist as free metal ions in the emulsions capable of reacting with the hydroperoxides to propagate oxidation. Earlier results obtained by Deiana et al. (*37*) and Gülçin (*5*) showed that caffeic acid was capable of reducing Fe^{3+} back to Fe^{2+}, thereby propagating lipid oxidation. Moreover, results obtained by Brenes-Balbuena et al. (*38*) and Garcia et al. (*39*) have shown that caffeic acid oxidized in the presence of iron. This might explain the increased lipid oxidation in the presence of caffeic acid compared to the other phenolics. Moreover, Keceli and Gordon have concluded that the *o*-diphenols in olive oil are active as antioxidants in oil and emulsions, but in the presence of iron, decomposition or oxidation of *o*-diphenols and reduction of Fe^{3+} to Fe^{2+} were more significant than chelation of iron by the *o*-diphenols (*40*). This may explain why rutin promoted oxidation more than naringenin.

Without iron at pH 3, only the emulsion with caffeic acid had a significant antioxidative effect (PV concentration) and a significant prooxidative effect (concentration of volatiles) compared to emulsions with the other phenolics or the emulsion without phenolics. This finding suggests that caffeic acid may also reduce low levels of endogeneous iron present in the fish oil or emulsifier.

At pH 6, all of the tested phenolic compounds interacted with iron, and for some of the compounds this interaction may have resulted in the formation of an iron−polyphenol complex and nanoparticles as suggested above. It may be speculated that the interaction between iron and the phenols may prevent them from acting as free radical scavengers. However, the finding that the phenolic compounds generally reduced the formation of peroxides and free radicals compared to the control at this pH in both Citrem and Tween emulsions with iron suggests that the phenolic compounds still had free radical scavenging activities despite the observed interaction with iron. In emulsions in which iron catalyzes peroxide decomposition and thereby decreases PV, a simultaneous increase in volatiles may be expected. Addition of the phenolic compounds decreased PV without increasing the formation of volatiles in the Citrem emulsions irrespective of the type of phenolic compound, but not in the Tween emulsions in the presence of caffeic acid (**Figure 7C**). Taken together these findings may suggest that the combined ability of both Citrem and the polyphenols to form complexes with iron prevented iron from decomposing peroxides. Moreover, it also seemed that caffeic acid lost its ability to reduce Fe^{3+} to Fe^{2+} when Citrem was used, but not when Tween was used. This might be due to the metal chelating properties of Citrem.

Without iron all of the different phenolic compounds tested at pH 6 had a significant antioxidative effect based on the concentration of peroxides and radicals. However, on the basis of the concentration of volatiles, caffeic acid acted as prooxidant and the other phenols had no significant effect on the formation of volatile oxidation products. The prooxidative effect of caffeic acid may be due to its ability to reduce endogeneous Fe^{3+} present in the fish oil or the emulsifier to Fe^{2+}. The lack of an effect of the other phenols on volatile formation suggests that these phenols are not as active as caffeic acid toward iron. It could also partly be due to a low level of oxidation in these emulsions, which made it difficult to detect any effect of the phenolic compounds.

In summary, the data showed that different analytical methods used to evaluate lipid oxidation showed different effects of the same antioxidant. However, irrespective of the analytical method it was found that when iron was present, the pH was crucial for the progress of lipid oxidation. At pH 3 the phenolic compound may be more active in reducing Fe^{3+} to Fe^{2+} than at pH 6, and this increased lipid oxidation at pH 3.

The data also showed that the physical location of the antioxidants may not be the only important factor governing the efficacy of the antioxidants in emulsions in which oxidation is catalyzed by iron as the only significantly surface active polyphenol, naringenin, gave very weak antioxidant activity. Moreover, the most water-soluble compound, caffeic acid, showed different effects depending on pH and emulsifier type. Thus, it was a strong prooxidant at pH 3 (with or without iron), but at pH 6 its effect depended on the emulsifier type and on the presence of iron. When Tween was used as an emulsifier, caffeic acid promoted formation of volatiles, but when Tween was replaced by Citrem, caffeic acid had no effect on volatile formation in emulsions with iron, and in emulsions without iron, it even seemed to have a slight antioxidative effect (**Figure 7D**). Hence, other factors such as (i) the electrical charges of the emulsifier and the antioxidant, (ii) the ability of the antioxidants to form complexes with iron and/or reduce Fe^3 to Fe^{2+}, which seemed to be particularly important for the prooxidative activity of caffeic acid, (iii) the physical structure of the emulsifier, which may influence the ability of antioxidants and iron to interact, and (iv) the ability of the emulsifier to chelate metal ions may also be important. Thus, the "polar paradox" hypothesis seems to be too simple to explain the complex mechanisms of antioxidant efficacy in multiphase systems in which oxidation is catalyzed by iron. The results indicated that phenol−iron complexes/nanoparticles were formed at pH 6, but further studies are necessary to elucidate their effect on lipid oxidation.

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