Effects of Lactoferrin, Phytic Acid, and EDTA on Oxidation in Two Food Emulsions Enriched with Long-Chain Polyunsaturated Fatty Acids

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The influence of the addition of metal chelators on oxidative stability was studied in a milk drink and in a mayonnaise system containing highly polyunsaturated lipids. Milk drinks containing 5% (w/w) of specific structured lipid were supplemented with lactoferrin (6–24 μM) and stored at 2 °C for up to 9 weeks. Mayonnaise samples with 16% fish oil and 64% rapeseed oil (w/w) were supplemented with either lactoferrin (8–32 μM), phytic acid (16–124 μM), or EDTA (16–64 μM) and were stored at 20 °C for up to 4 weeks. The effect of the metal chelators was evaluated by determination of peroxide values, secondary volatile oxidation products, and sensory analysis. Lactoferrin reduced the oxidation when added in concentrations of 12 μM in the milk drink and 8 μM in the mayonnaise, whereas it was a prooxidant at higher concentrations in both systems. In mayonnaise, EDTA was an effective metal chelator even at 16 μM, whereas phytic acid did not exert a distinct protective effect against oxidation. The differences in the equimolar effects of the metal chelators are proposed to be due to differences in their binding constants to iron and their different stabilities toward heat and low pH.

KEYWORDS: Lactoferrin; EDTA; phytic acid; structured lipids; fish oil; mayonnaise; milk drink

INTRODUCTION

Lipid oxidation is a considerable problem in lipid-bearing foods, especially in food products containing lipids with highly polyunsaturated fatty acids (PUFA). Lipid oxidation of foods containing these lipids takes place almost instantly unless careful precautions are taken. Particular problems arise when the highly unsaturated oils are emulsified into various food systems (*1*). The so-called specific structured lipids produced by enzymatic interesterification to possess specific nutritional and functional properties have a lower oxidative stability than traditional lipids (*2*, *3*). The oxidation of *n*−3 PUFA in emulsions furthermore gives rise to particularly unpleasant fishy and rancid off-flavors (*4*, *5*). Moreover, reactive aldehydes formed as a result of lipid oxidation have been suggested to be involved in processes leading to cardiovascular diseases (*6*). The successful incorporation of *n*−3- and/or *n*−6-rich oils or specifically structured, unsaturated lipids into foods for nutritional functionality therefore requires efficient protection against oxidative flavor deterioration of the lipids. Unfortunately, conventional chain-breaking antioxidant systems, including, for example, various tocopherol systems and ascorbic acid, do not provide sufficient oxidative protection of the lipids in these systems (*7*).

Recently, we reported that addition of calcium disodium ethylenediaminetetraacetic acid (EDTA) could reduce lipid oxidation in fish oil enriched milk (*5*), which suggests that metal ions may play an important role in the oxidative deterioration of fish oil enriched milk. Likewise, lipid oxidation and formation of off-flavors were efficiently blocked in mayonnaise by the addition of 75 mg/kg (equivalent to 200 μM) EDTA (*4*). This finding corroborated our previously suggested hypothesis that iron stemming from egg yolk components located at the oil−water interface is the main catalyst of oxidation in fish oil enriched mayonnaise (*8*, *9*). It was proposed that iron catalyzes oxidation by breaking down pre-existing lipid hydroperoxides that will give rise to formation of alkoxyl radicals. These radicals can either react with intact lipid molecules, and thereby further propagate oxidation, or further decompose to secondary volatile oxidation products, which will lead to the formation of undesirable off-flavors. However, the effects of lower concentrations of EDTA have not been examined. EDTA is a diaminotetraacetic acid compound (**Figure 1**) with a high metal chelating activity and a reported binding constant for a ferric−EDTA complex of 1.3×10^{25} (*10*). However, EDTA is a compound produced by chemical synthesis and is therefore categorized as "a synthetic compound". The food industry, as well as consumers, often wish to replace synthetic compounds with natural ones with a more "green" image. Lactoferrin and phytic acid (**Figure 1**) are examples of natural compounds with metal chelating properties.

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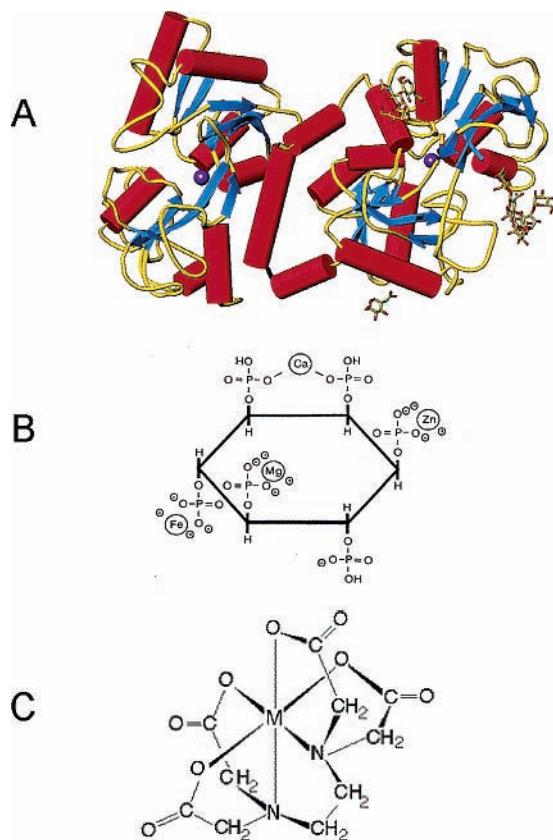† Danish Institute for Fisheries Research.
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Figure 1. Structures for (**A**) lactoferrin[1], (**B**) phytic acid,[2] and (**C**) EDTA[3]. (**A**) From Stanley Moore, Department of Biochemistry, University of Saskatchewan, Saskatoon, Canada; (**B**) http://www.ansc.purdue.edu/courses/ansc443/images/Class_notes/Nutrition/Phytic.jpg&imgrefurl=http://www.ansc.purdue.edu/courses/ansc443/Class_notes/Nutrition.html&h=1198&w=1970&sz=233&tbnid=sQ69W_f5IasJ:&tbnh=91&tbnw=149&start=1&prev=/images%3Fq%3Dphytic%2Bacid%26hl%3Dda%26lr%3D%26ie%3DUTF-8%26oe%3DUTF-8,100504); (**C**) (http://www.tu-bs.de/institute/ibvt/forschung/projekte/p1-d.htm, 100504).

Lactoferrin is a milk glycoprotein occurring naturally in numerous bodily secretions, including milk, tears, mucus, blood, and saliva. This presence suggests a role in nonspecific defense against invading pathogens. Lactoferrin is also the main iron-bearing protein in cow's milk, and it is able to bind two Fe^{3+} in cooperation with two HCO_3^- ions when fully saturated (*11, 12*). Lactoferrin, purified from cow's milk, has been shown to have a strong antioxidative effect when used in concentrations of $1-20\ \mu M$ in 10% oil-in-water model emulsions and in infant formulas, respectively (*11, 13*). However, the lactoferrin addition did not affect lipid oxidation in either mayonnaise ($10\ \mu M$ lactoferrin) (*3*) or the milk drink ($12.5\ \mu M$ lactoferrin) (*2*) that had been enriched with structured oil. However, only one concentration of lactoferrin was tested in each of these emulsions, and the metal saturation of the lactoferrin could not be assessed. It can therefore not be excluded that lactoferrin may exert an antioxidative effect in PUFA-enriched mayonnaises and milk drinks if employed at other concentrations.

Phytic acid is a natural plant inositol hexaphosphate constituting 1−5 wt % of many cereals and legumes (*14*), and it forms salts with divalent cations. Phytic acid has been shown to prevent lipid oxidation in 50% soybean oil-in-water emulsions (1 mM phytic acid) and in cooked minced chicken breasts (0.75−3.5 mM phytic acid) (*14*). To our knowledge the ability of phytic

Table 1. Experimental Design of Milk Drink and Mayonnaise Experiment

	code[a]	concn in mg/kg	concn in μM	metal chelator/ Fe ratio[b]
		Milk Drink		
lactoferrin	LM0	0	0	
	LM6	500	5.6	2:1
	LM12	1000	11.1	4:1
	LM18	1500	16.7	6:1
	LM24	2000	22.2	8:1
		Mayonnaise		
no metal chelator	NoM	0	0	
lactoferrin	L8	707	7.9	$^1/_4$:1
	L16	1413	15.7	$^1/_2$:1
	L24	2120	23.6	$^3/_4$:1
	L32	2826	31.4	1:1
phytic acid	P16	15	15.7	$^1/_2$:1
	P94	87	94.2	3:1
	P126	116	125.6	4:1
EDTA	E16	6	15.7	$^1/_2$:1
	E 64	24	62.8	2:1

[a] The number in the code name refers to the amount (μM) of metal chelator added. [b] The metal chelator/Fe ratios (mol/mol) were calculated on the basis of previous determinations of the iron content in mayonnaise and milk. Milk and mayonnaise contained 2.9 and 31.4 μM Fe, respectively (*37*).

acid to prevent lipid oxidation has never been investigated in a real food emulsion such as fish oil enriched mayonnaise.

The aims of the present study were therefore to determine the antioxidative effect of lactoferrin in a strawberry-flavored milk drink containing 5% (w/w) of specific structured lipids based on sunflower oil and in fish oil enriched mayonnaise [16% of fish oil and 64% (w/w) of rapeseed oil] as well as to determine the effect of phytic acid and low concentrations of EDTA in the mayonnaise system. This experimental design was driven by the hypothesis that metal chelators are efficient in preventing lipid oxidation in these systems due to their ability to chelate and inactivate metal ions that may otherwise catalyze oxidative flavor deterioration as described above. However, the efficacy of the metal chelators may depend on their metal-binding properties and other physicochemical parameters, and therefore we wished to compare different types of metal chelators.

In the milk drink, concentrations of lactoferrin up to 24 μM were employed to investigate the effect of concentrations both higher and lower than the 1000 mg/kg (12 μM) used in our previous experiments (*15*). Because the antioxidative effect of EDTA has been suggested to depend on the ratio between EDTA and iron (*16*), we based the selection of metal chelator concentrations in the mayonnaise on calculations of the ratio between metal chelator and iron concentrations as indicated in **Table 1**. For the sake of comparison, similar metal chelator/iron ratios were employed with the other metal chelators and in the milk drink.

MATERIALS AND METHODS

Materials. Specific structured lipid based on sunflower oil and caprylic acid was produced as reported previously (*17*) with the following composition of fatty acids: 38% C8:0, 3% C16:0, 2% C18:0, 14% C18:1n−9, and 42% C18:2n−6. The rest of the fatty acids were equal to or below 0.3%. The contents of α- and γ-tocopherol in the specific structured oil used for the milk drink were 56 and 1 $\mu g/g$, respectively. The low levels are due to the removal of tocopherols during purification of the triacylglycerol after interesterification. Refined unhydrogenated rapeseed oil was from Århus United A/S (Århus, Denmark) with the following content of unsaturated fatty acids: 60.3%

C18:1, 20.6% C18:2, 9.0% C18:3, 1.5% C20:1; tocopherol content was 450 μg/g; peroxide value (PV), 0.3 mequiv/kg; anisidine value (AV), 1.7; free fatty acids (FFA), 0.10%; iron, <0.1 μg/g; and copper, <0.05 μg/g. Unrefined fish oil (from sand eel) was obtained from TripleNine Fish Protein amba (Esbjerg, Denmark). The fish oil was refined and deodorized at the pilot plant of BioCentrum, Technical University of Denmark, with the following content of unsaturated fatty acids: 5.5% C16:1, 10.3% C18:1, 2.0% C18:2, 1.8% C18:3, 4.6% C18:4, 6.4% C20: 1, 10.3% C20:5, 10.8% C22:1, 10.8% C22:6; tocopherol content was <100 μg/g; PV, 0.1 mequiv/kg; AV, 3.2; FFA, 0.10%; iron, <0.1 μg/g; and copper, <0.05 μg/g. Skimmed milk was from Arla Foods A/S (Brabrand, Denmark), and sugar was purchased from Danisco (Copenhagen, Denmark). Egg yolk with 3% (w/w) of salt (NaCl) was from Danæg (Copenhagen, Denmark), potassium sorbate from Merck (Darmstadt, Germany), tarragon vinegar (7% w/w) from Lagerberg (Hamburg, Germany), and EDTA (calcium disodium ethylenediaminetetraacetate) from Sigma (Steinheim, Germany). Strawberry flavoring 11088 (natureidentical), Recodan emulsifier (mono- and diglycerides of fatty acids, carageenan, and guar gum), and Grindsted FF 5105 stabilizer (guar gum and sodium alginate) were donated by Danisco (Brabrand, Denmark). Lactoferrin was donated by DMV International (Veghel, The Netherlands). Phytic acid was from Sigma (St. Louis, MO). Other chemicals and solvents were of analytical grade.

Milk Drink Production. Five different milk drinks based on skimmed milk were produced. The composition of the milk drinks was 5.0% lipid (specific structured lipid based on sunflower oil and caprylic acid), 5.0% sugar, 0.2% Recodan emulsifier, and 0.13% strawberry flavoring (all percentages are w/w). Metal chelator (0−2000 mg/kg lactoferrin) was added to the milk drink during production (see **Table 1**). The amount of lactoferrin originally present in milk is ∼0.2 mg/kg (*18*), and this level was considered to be insignificant compared to the added amounts. The amount of skimmed milk was adjusted so that the total amount of ingredients including milk added up to 2000 g in all experiments. Milk drinks were produced as previously described (*2*). The milk drink was exposed to "high-temperature−short time" (HTST) treatment before it was poured into sterile bottles.

Mayonnaise Production. Mayonnaises were produced in 500 g batches composed of 16% fish oil, 64% rapeseed oil, 4.0% tarragon vinegar, 4.0% egg yolk, 0.3% NaCl, 1.0% sugar, 0.1% potassium sorbate, and 0.2% Grindsted FF5105 (all percentages are w/w). Metal chelators [EDTA, lactoferrin, phytic acid (**Table 1**)] were added to the aqueous phase before mayonnaise production. The amount of water was adjusted so that the total weight of ingredients and water added up to 500.0 g in all experiments. Mayonnaises were produced as previously described (*19*). The pH of the mayonnaises was ∼4.

Storage Experiments. Milk drinks were stored in sterile glass bottles with screw lids in the dark at 2 °C for 9 weeks with samples taken for analyses at 0, 2, 4, 6, 8, and 9 weeks. Mayonnaises were stored in 50 mL capped, brown glass jars in the dark for 4 weeks at 20 °C, and samples were taken at 0, 1, 2, 3, and 4 weeks.

The sensory evaluation was carried out immediately after sampling (see below) while the parallel bottles with milk drink or jars with mayonnaise were frozen at −30 °C until determination of PV and secondary volatile oxidation products. Replicates were withdrawn from the same bottle or jar.

Extraction of Lipid. Lipids were extracted from thawed milk drinks according to the method of Bligh and Dyer (B&D) (*20*) using 15 g of sample, 30 mL of methanol, and 30 mL of chloroform. The solution was centrifuged and the extract filtered through a phase separator filter (Advantech, Tokyo, Japan). Ten grams of the B&D extract was evaporated to calculate the percentage of oil gravimetrically. Mayonnaises were thawed and centrifuged at 25400*g* for 10 min. The separated oil was used for the analysis of peroxide values. Extractions were performed in duplicate.

Determination of Primary Oxidation Products by PV. PV was determined on the oil phase separated from the mayonnaise or the B&D extract from the milk drink according to the International Dairy Federation (IDF) method described by Shantha and Decker (*21*). The absorption was measured on a Shimadzu UV-160 spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD). Analyses were made in triplicate.

Determination of Volatile Secondary Oxidation Products. Four grams of frozen milk drink or mayonnaise was weighed into a pearshaped glass flask together with *n*-dodecane as an internal standard, and headspace volatiles were collected on Tenax tubes by purging the samples with nitrogen as previously described (*2*, *22*). Volatile acids were removed from the mayonnaise but not the milk drink by neutralization with potassium hydroxide during the dynamic headspace collection. Trapped volatiles were separated and quantified by gas chromatography (GC) on a Hewlett-Packard 5890 (Avondale, PA) gas chromatograph equipped with a flame ionization detector (FID) and analyzed as previously described (*2*, *22*). Identification of compounds was done by retention time index and confirmed by GC-MS and by spiking with external standards. Results from the analyses are given as nanograms per gram or peak area per gram of milk drink/mayonnaise. Analyses were made in triplicate. The inhibitory effect of a metal chelator on the production of volatile compounds is given as percent inhibition and was calculated as

$$\frac{(\text{level in sample with metal chelator} - \text{level in reference}) \times 100\%}{\text{level in reference}}$$

where the reference is the corresponding sample (to each different product mayonnaise or milk drink) without metal chelator added.

Sensory Assessment (Milk Drink Only). Eight trained assessors ranked the five milk drinks on four attributes: fishy and strawberry aroma (smell) and flavor (taste). The five milk drinks were served simultaneously in portions of 35 mL (5 °C) in transparent capped plastic jars. Samples were blinded. Distilled water and crisp bread were provided for oral rinsing at the beginning of sessions and when needed. Milk drinks were ranked on one sheet of paper with four tables, one for each attribute. Panel members were allowed to rank similar samples equally. Statistically significant differences were calculated by a nonparametric Friedman-type statistic algorithm as described by Meilgaard et al. (*23*).

Data Analysis. *Milk Drink.* PV, volatiles data, and sensory results from the storage experiment were analyzed by multivariate data analysis using The Unscrambler, version 7.6, software program (CAMO). All data were analyzed by analysis of variance partial least-squares regression (APLSR) using design variables as *X* data and the measured variables as *Y* data. The design variables for lactoferrin concentration were LM0, LM6, LM12, LM18, and LM24 and for replicates, Rep1, Rep2, and Rep3 (for explanation of code names please refer to **Table 1**). Cross-validation on treatments was used to validate the APLSR model. All variables were weighed by 1/standard deviation. 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 (*24*).

Mayonnaise. Data obtained from the storage experiment (PV and volatile secondary oxidation products) were analyzed as described above except that the design variable for reference without metal chelator was NoM and variables for metal chelator type and concentration were E16, E64, L8, L16, L24, and L32 and P16, P94, and P126 (for explanation of code names please refer to **Table 1**). Full cross-validation was used to validate the APLSR model.

Differences between milk drinks or mayonnaises during storage were also analyzed using ANOVA (GraphPad Prism 4.0; San Diego, CA). Differences were considered to be significant at $p < 0.05$.

RESULTS

First, we compared the effects of lactoferrin in the milk drink and in the mayonnaise. To assist with this comparison two APLSR models were computed: model 1, data from the milk experiment; model 2, data obtained from mayonnaise experiment from the sample without metal chelator and from the four samples containing lactoferrin. Second, the effects of lactoferrin, phytic acid, and EDTA in mayonnaise were compared at equimolar levels and at other different addition levels. For this

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Table 2. Peroxide Values during Storage (2 °C) of Milk Drinks and Significant Regression Coefficients (RC) for Weeks 6 and 8[a]

week	LM0	LM6	LM12	LM18	LM24	SD
0	0.94	0.83	1.19	0.82	0.92	0.25
2	0.63	0.81	1.14	0.88	1.11	0.20
4	0.58a	0.83ab	1.22ab	1.23ab	1.44 b	0.17ab
6	1.25	1.23	1.78	1.60	1.52	0.18
8	1.04a	1.23a	1.45ab	1.40ab	1.90b	0.08
9	1.28a	1.13a	1.46ab	1.59ab	2.04b	0.05
RC	−	− −	+		+	

[a] Values are calculated as mequiv of peroxide/kg ($n = 3$), and SD gives the pooled standard deviation for all samples in one week. The number after LM refers to the amount of lactoferrin added in μM. RC indicates significant regression coefficients for weeks 6 and 8. + indicates that the regression coefficient was significantly positive and − that it was significantly negative in one of the weeks. ++ or − − indicates that the regression coefficients were significantly positive or negative at the two sampling times indicated ($p < 0.05$). Different letters within a row indicates that mean values were significantly different ($p < 0.05$).

Table 3. Peroxide Values and APLSR Analysis Regression Coefficients (RC2 and RC3) of Mayonnaises during Storage at 20 °C[a]

week	NoM	L8	L16	L24	L32	P16	P94	P126	E16	E64
0	0.59f	0.44bc	0.39a	0.40a	0.42ab	0.47c	0.43bc	0.45c	0.54e	0.49d
1	1.23g	0.72c	1.01e	1.49 j	0.78d	1.26h	1.46i	1.19f	0.59b	0.55a
2	5.67f	2.98c	6.33e	8.16j	4.90d	7.27g	7.57h	7.82i	0.98c	0.88a
3	14.40f	10.82d	15.98h	16.29i	19.30j	15.65g	9.68c	13.99e	1.88i	1.10a
4	16.86h	6.83c	13.72e	14.77f	15.41g	6.80c	10.19d	20.90i	3.75b	1.16a
RC2		− −				nd	nd	nd	nd	nd
RC3	+		+						−	− −

[a] Values are calculated as mequiv of peroxide/kg and given as mean ($n = 3$). E, EDTA; P, phytic acid; L, lactoferrin (the number following the letter indicates the amount of metal chelator (μM) added to the mayonnaise); RC2, significant regression coefficients for week 3 and 4 obtained from model 2; RC3, RC from model 3; for interpretation of + and −, please refer to **Table 2**; nd, not determined. Different letters within a row indicate that mean values were significantly different ($p < 0.05$).

comparison an APLSR model (model 3) on all data from the mayonnaise experiment was computed.

Sensory Data. The most characteristic sensory descriptors in the milk drinks, strawberry aroma (smell) and flavor (taste) as well as rancid aroma and flavor, were ranked by increasing intensity at the beginning of the experiment and after 6 and 9 weeks of storage. The averages for the four descriptors strawberry smell, rancid smell, strawberry aroma, and rancid aroma for all assessors at all weeks were 3.0 with standard deviations of 0.5, 0.4, 0.6, and 0.6 for the four descriptors, respectively (data not shown). Thus, no significant differences were observed for any of the sensory descriptors among the five milk drinks. This lack of sensory difference was probably due to a relatively low degree of oxidation in all samples as will be discussed in the following.

Primary Oxidation Products. *Milk Drink.* Addition of 12 μM or greater lactoferrin apparently had a prooxidative effect on PV formation in the milk drink as the PV were highest in LM24 followed by LM18 and LM12 after 8 and 9 weeks of storage (**Table 2**). This prooxidative tendency became most apparent late during the storage period, and addition of 24 μM lactoferrin resulted in significantly higher PV than did the addition of 0 or 6 μM lactoferrin after 8 weeks of storage (**Table 2**). Furthermore, the regression coefficients obtained from the APLSR model on the data from the milk drink after 6 and 8 weeks were negative for 0 and 6 μM lactoferrin (LM0 and LM6) but positive for the samples with 12 or 24 μM lactoferrin, LM12 and LM24 (**Table 2**). A positive regression coefficient indicates that the design variable has a positive effect on the measured variable (and, analogously, a negative regression coefficient indicates a negative influence). In this case the positive regression coefficients obtained with LM12 and LM24 therefore corroborated that lactoferrin addition exerted a prooxidative effect on the formation of PV. Because the PV of the reference sample (LM0) was also weighed in the APLSR model (that included all of the measured oxidation variables), the negative regression coefficients of LM0 and LM6 must be interpreted as relative to the high LM dose samples, and in this case the negative regression coefficients therefore indicate a "null effect" rather than an "antioxidative effect" of the LM6 addition, corroborating the interpretation that addition of high levels (>12 μM lactoferrin) exerted a prooxidant effect. Interestingly, PV did not increase to more than 2 mequiv/kg in any of the milk drinks during the 9 weeks of storage.

Mayonnaise. Similarly, the APLSR model on the lactoferrin data in mayonnaise revealed a negative regression coefficient

(RC2) for the PV at the lowest lactoferrin concentration (8 μM) (**Table 3**). The data shown in **Table 3** confirmed that the mayonnaise with 8 μM lactoferrin had lower PV during storage than the mayonnaise without metal chelator. In contrast, addition of lactoferrin at higher concentrations (16−32 μM) resulted in PV at approximately the same levels as those of the mayonnaise without metal chelator throughout the storage period.

The regression coefficients from the APLSR model on all mayonnaise samples (RC3) were also studied (**Table 3**). The significant negative regression coefficient for L8 confirmed that the addition of 8 μM lactoferrin slightly reduced PV in fish oil enriched mayonnaise (**Table 3**). The significant positive regression coefficient for the PV in L16 analogously confirmed the prooxidative effect of this addition level of lactoferrin. A very strong antioxidative effect of EDTA in mayonnaise was also found. Thus, PV had significantly negative regression coefficients after 3 and 4 weeks for both EDTA concentrations (**Table 3**). Phytic acid, on the other hand, had no significantly positive or negative regression coefficients and thus did not influence the development of peroxides in the fish oil enriched mayonnaise. By observing the raw PV data from weeks 1−4, we could confirm that the mayonnaise with the lowest lactoferrin concentration (8 μM) generally had lower PV than both the control without metal chelator addition and the other mayonnaises containing lactoferrin or phytic acid. PV was lowest in the E64 mayonnaise and second lowest in the E16 mayonnaise, and both had much lower PV (max PV = 1.9 and 3.8 mequiv/kg) than the other mayonnaises (max PV between 10.2 and 20.9) (**Table 3**). The raw data also showed that PV for the mayonnaise with 94 μM phytic acid were lower than PV in the sample without metal chelator in the later part of the storage period (**Table 3**). In contrast, the two other concentrations of phytic acid generally resulted in PV at the same level as the sample without metal chelator except in week 4, when P16 had a lower PV and P126 had a higher PV.

In summary, the PV were lower in the milk drinks than in the mayonnaises. The PV in the milk drinks increased in the order LM0 ≈ LM6 < LM 12 ≈ LM18 < LM24. In the mayonnaises the order of the PV was E16 < E64 ≪ L8 < P94 < P16 ≈ L16 ≈ L24 ≈ L32 ≈ NoM ≈ P126.

Volatile Secondary Oxidation Products. *Milk Drinks.* Twenty-six volatile secondary oxidation products from the milk drinks were identified. Thirteen of these volatiles originated from the milk and the added strawberry aroma. The levels of these compounds were similar for all milk drinks and did not change during storage (data not shown), and they were therefore omitted from further analysis. The regression coefficients obtained from

Table 4. Significant Regression Coefficients for Secondary Volatile Oxidation Products in Milk Drinks after 6 and 8 Weeks (Storage at 2 °C)[a]

	regression coefficients				
	LM0	LM6	LM12	LM18	LM24
2-propanone	++	+		+	−
2-butanone			−		+
pentylfuran	++	−	−		−
1-butanol	++	++	−	+ −	−
pentanal	−		− −	+	++
2,3-pentadione	+	+		− −	−
1-pentanol	−			+	
hexanal	−	−	− −	++	++
heptanal			− −		++
2-heptenal	−	−	−	+	++
octanal				−	
2-methyl-3-ethyl-1,3-hexadiene	+		− −		+
2-octenal	−		−		++

[a] For interpretation of + and −, please refer to **Table 2**. A blank entry indicates that the design variable was not significant.
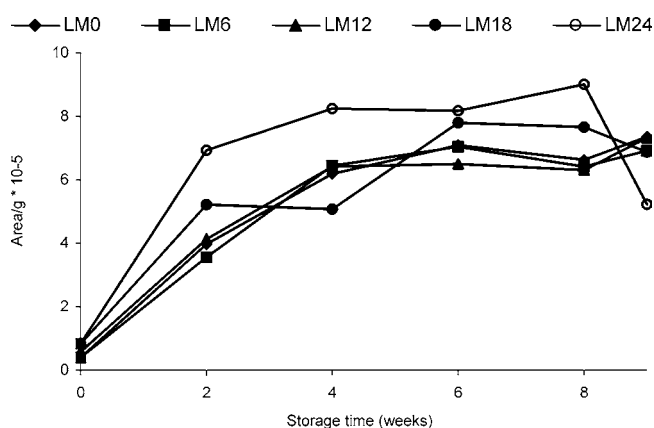
Figure 2. Development of hexanal in milk drinks during storage at 2 °C for up to 9 weeks. For interpretations of code names please refer to **Table 1**.

the APLS model for the design variables of the remaining 13 volatile compounds present in the milk drinks are shown in **Table 4**. After 6 weeks of storage, the main effect of adding 12 μM lactoferrin was negative for 10 of the 13 compounds. On the contrary, the main effect of the other lactoferrin concentrations was positive for 2, 4, 5, and 6 compounds in the milk drinks containing 6, 18, 0, and 24 μM lactoferrin, respectively. Interestingly, the compounds that correlated posi-

tively with LM24 were mainly aldehydes, whereas the compounds that correlated positively with LM6, LM18, and LM0 were ketones, furan, alcohol, and aldehydes (**Table 4**). In week 8, the pattern was less clear, with four negative regression coefficients for LM12 and between two and four negative or positive regression coefficients for LM0, LM6, and LM18. Conversely, the effect of adding 24 μM lactoferrin was more pronounced in week 8. There were six positive regression coefficients as in week 6, but only one negative regression coefficient compared to three in week 6 for the LM24 design variable. Four aldehydes (pentanal, hexanal, 2-heptenal, and 2-octenal) were present in higher levels in LM24 compared to the other milk drinks during storage. This is exemplified by hexanal in **Figure 2**. Eight volatiles developed similarly in all of the milk drinks. The ketones (2-propanone and 2-butanone) and pentylfuran developed slowly (data not shown). On the contrary, the levels of 2,3-pentadione, 1-pentanol, heptanal, octanal, and 2-methyl-3-ethyl-1,3-hexadiene increased more abruptly and then leveled out. The concentrations of selected volatiles were calculated, and the results from week 6, when concentrations were highest, are listed in **Table 5**. These results confirm that the concentrations were generally highest in LM24 (four of six compounds) and lowest in LM12, whereas the effects obtained with 6 and 18 μM lactoferrin were less clear. The percent inhibition data showed that 12 μM lactoferrin was most efficient in inhibiting the formation of volatiles (10 of 13 compounds), whereas 24 μM was prooxidative for 7 of 13 compounds (**Table 5**). Addition of 12 μM lactoferrin resulted in up to 76% inhibition for 1-butanol, but the formation of the other compounds was inhibited by only between 2 and 42%.

Taken together, the PV and volatiles data indicated the following order of oxidative stability of the milk drink samples supplemented with lactoferrin: LM24 < LM0 ≈ LM6 ≈ LM18 < LM 12.

Mayonnaise. On the basis of our previous studies on fish oil enriched mayonnaise (*8*), eight volatiles from the mayonnaise samples were selected for quantification (**Tables 6** and **7**) as the concentration of these volatiles has been shown to correlate with fishy off-flavor formation. Similar to the concentration effect of lactoferrin in the milk drink, we observed that in mayonnaise, L24 had significantly positive regression coefficients for six of eight volatiles in either week 3 or 4 or in both weeks (**Table 6A**), whereas L8 had significantly negative regression coefficients for seven of eight volatiles (**Table 6A**). In contrast, L16 and L32 had no or only one negative regression coefficient in model 2 (**Table 6A**). These data therefore suggest that addition of 8 μM lactoferrin slightly reduced the formation

Table 5. Concentration of Volatiles in Milk Drinks after 6 Weeks of Storage and Percent Inhibition in Week 6[a]

	concentration (ng/g)					% inhibition			
	LM0	LM6	LM12	LM18	LM24	LM6	LM12	LM18	LM24
2-propanone	nd	nd	nd	nd	nd	31	25	−12	11
2-butanone	nd	nd	nd	nd	nd	12	23	8	−4
pentylfuran	54 ± 4	34 ± 1	33 ± 1	35 ± 2	34 ± 2	39	42	36	40
1-butanol	nd	nd	nd	nd	nd	−7	76	22	75
pentanal	219 ± 11	231 ± 12	194 ± 7	242 ± 12	261 ± 19	−5	10	−9	−15
2,3-pentadione	nd	nd	nd	nd	nd	8	15	25	13
1-pentanol	nd	nd	nd	nd	nd	−60	−73	−32	−55
hexanal	2418 ± 145	2400 ± 184	2216 ± 55	2659 ± 53	2788 ± 52	1	8	−10	−15
heptanal	45 ± 3	44 ± 3	39 ± 1	42 ± 1	46 ± 1	2	17	7	−4
2-heptenal	165 ± 32	175 ± 35	163 ± 15	186 ± 7	209 ± 4	−5	1	−10	−21
octanal	91 ± 14	91 ± 13	81 ± 5	83 ± 5	85 ± 1	0	11	9	7
2-methyl-3-ethyl-1,3-hexadiene	nd	nd	nd	nd	nd	4	18	12	4
2-octenal	nd	nd	nd	nd	nd	−1	2	−8	−27

[a] Concentrations given as mean ($n = 3$) ± SD; percent inhibition calculated on the basis of peak areas; nd, calibration curves not available.

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Table 6. Regression Coefficients for Volatile Secondary Oxidation Products after 3 and 4 Weeks of Storage from APLSR Models 2 and 3[a]

(A) Sample without Metal Chelator and the Four Mayonnaises with Lactoferrin in Different Concentrations

	NoM	L8	L16	L24	L32
1-penten-3-one	− −	−		++	
pentanal		−			
2-E-pentenal		−		++	
2-E-hexenal		−		+	−
heptanal	+			+	
2-pentylfuran	− −	−		++	
2-E-heptenal			− −	+	
2,4-E,E-heptadienal		−			

(B) Based on All Mayonnaise Samples

	NoM	L8	L16	L24	L32	P16	P94	P126	E16	E64
1-penten-3-one	+			++			+	+	− −	− −
pentanal				+			+		− −	− −
2-E-pentenal				++			+		− −	− −
2-E-hexenal	++			++					−	−
heptanal	++			+					− −	− −
2-pentylfuran			+	+					− −	− −
2-E-heptenal	+								− −	− −
2,4-E,E-heptadienal									− −	− −

[a] For interpretation of + and −, please refer to **Table 2**. A blank entry indicates that regression coefficients were not significant in any of the weeks.

of most volatiles compared to no addition, whereas addition of 24 μM slightly increased the formation of most volatiles. Addition of 16 and 32 μM lactoferrin did not significantly influence volatile formation. Taken together, the PV and volatiles data indicated the following order of stability for fish oil enriched mayonnaise with lactoferrin: L24 < NoM $\approx$ L16 $\approx$ L32 < L8.

Interestingly, all volatiles had significantly negative regression coefficients for both EDTA concentrations after 3 and 4 weeks (**Table 6B**), indicating significantly protective effects of the EDTA additions. The effect of phytic acid was not clear as P94 had three significantly positive regression coefficients (1-penten-3-one, pentanal, and 2-E-pentenal) and P126 had one significantly positive regression coefficient for 1-penten-3-one. Taken

together, the data from the statistical model on all samples (model 3) and the model on the reference and lactoferrin samples (model 2) showed that the antioxidative effect observed for lactoferrin when added in a concentration of 8 μM was much weaker than the antioxidative effect of EDTA. Most of the regression coefficients from a model on the reference and phytic acid samples were not significant (data not shown). This observation indicates that phytic acid did not significantly affect lipid oxidation in mayonnaise.

To illustrate the effect of the various metal chelators on the development in volatiles, plots of 2-pentenal and 2-hexenal are shown as examples in **Figure 3**. These plots confirm that the mayonnaise with 8 μM lactoferrin had lower concentrations of the two volatiles compared with the mayonnaise without metal chelator, whereas the mayonnaise with 24 μM lactoferrin had higher concentrations. Mayonnaises with 16 and 32 μM lactoferrin were more similar to the reference. Moreover, the strong antioxidative effect of EDTA is also clearly seen. Concentrations of volatiles and percent inhibition of volatile formation after 3 weeks of storage are shown in **Table 7**. These data supported the above interpretation of **Table 6** and **Figure 3**. Hence, lactoferrin in a concentration of 24 μM clearly promoted volatile formation (percent inhibition between −121 and 16%), whereas 8 and 32 μM slightly inhibited volatile formation (percent inhibition between −7 and 36%). In the EDTA-supplemented samples, concentrations of the volatiles were much lower and the percent inhibition of volatiles formation was much higher compared to the other samples. EDTA thus inhibited volatile formation between 56 and 96%, and 16 μM EDTA seemed to inhibit volatile formation to the same extent as 64 μM EDTA. Phytic acid did not exert a clear effect.

Correlation Loadings Plot from Model 1. *Milk Drink.* To visualize the effects of the metal chelators on the different variables, the correlation loadings plots are shown in **Figure 4**. From the location of the design variables, it was obvious that principal component (PC) 1 described differences in the sensory and chemical data that changed with the lactoferrin concentration. PC2 described differences between the milk drinks containing 0 or 24 μM lactoferrin and the milk drink with 12 μM lactoferrin. In **Figure 4A**, the positive descriptors strawberry

Table 7. Concentration of Volatiles and Percent Inhibition of Volatile Formation in Mayonnaise after 3 Weeks of Storage

(A) Concentration of Volatiles (ng/g) with and without Metal Chelators[a]

	1-penten-3-one	pentanal	2-E-pentenal	hexenal	heptanal	pentylfuran	2-E-heptenal	2,4-E,E-heptadienal
NoM	94 ± 13	105 ± 10	133 ± 11	170 ± 7	39 ± 3	7 ± 1	70 ± 7	105 ± 31
L8	101 ± 9	74 ± 4	119 ± 12	136 ± 17	31 ± 5	7 ± 2	45 ± 3	100 ± 15
L16	106 ± 6	84 ± 1	149 ± 24	187 ± 22	35 ± 3	15 ± 2	62 ± 7	90 ± 4
L24	134 ± 5	196 ± 84	175 ± 16	199 ± 29	33 ± 4	16 ± 3	57 ± 3	121 ± 10
L32	101 ± 7	86 ± 10	117 ± 5	147 ± 21	28 ± 4	9 ± 2	49 ± 1	95 ± 19
P16	104 ± 2	105 ± 1	128 ± 2	153 ± 15	30 ± 2	7 ± 1	44 ± 2	79 ± 6
P94	106 ± 10	123 ± 7	119 ± 15	149 ± 6	30 ± 2	7 ± 0	47 ± 1	82 ± 4
P126	99 ± 10	119 ± 10	132 ± 15	185 ± 19	34 ± 1	11 ± 3	52 ± 2	87 ± 1
E16	18 ± 7	38 ± 2	20 ± 2	72 ± 6	5 ± 1	2 ± 1	18 ± 2	24 ± 18
E64	3 ± 10	5 ± 7	37 ± 9	51 ± 4	9 ± 0	3 ± 0	15 ± 2	29 ± 17

(B) % Inhibition of Volatile Formation by Addition of Metal Chelators[b]

	1-penten-3-one	pentanal	2-E-pentenal	hexenal	heptanal	pentylfuran	2-E-heptenal	2,4-E,E-heptadienal
L8	−7	29	10	20	21	0	36	4
L16	−13	20	−12	−10	11	−111	11	14
L24	−42	−86	−31	−17	16	−121	18	−15
L32	−7	19	12	14	30	−31	30	9
P16	−10	0	4	10	24	0	37	24
P94	−12	−17	10	12	25	−3	33	22
P126	−4	−13	1	−9	14	−58	25	17
E16	81	64	85	57	87	75	74	77
E64	96	96	72	70	78	56	79	73

[a] Concentrations given as mean ($n = 3$) ± SD. [b] Inhibitive effect of metal chelators on production of volatile compounds. Negative values indicate a prooxidative effect.
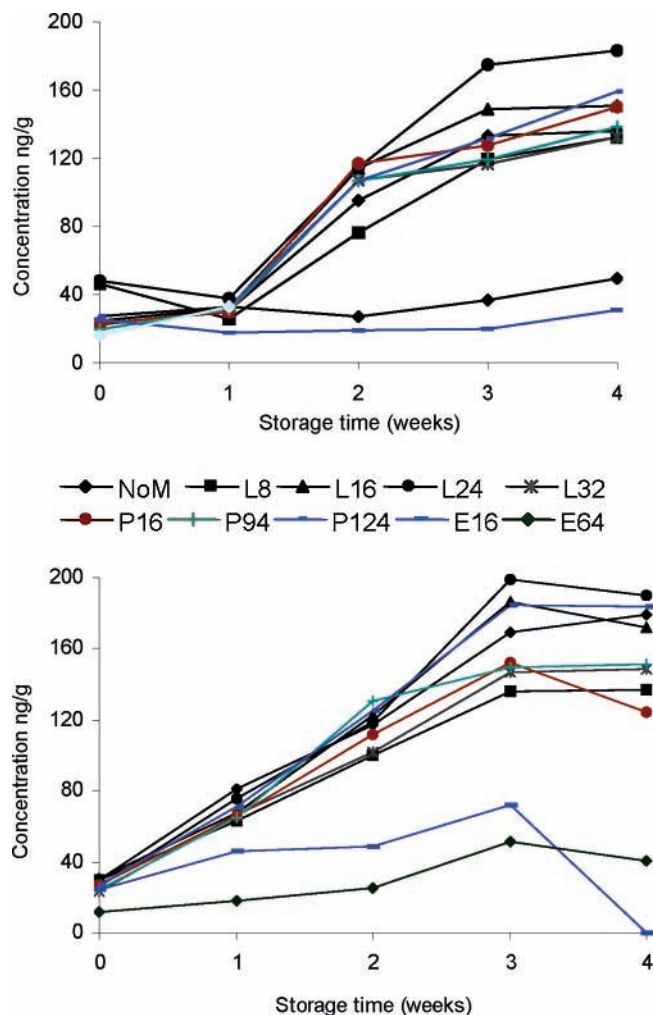
Figure 3. Development of (**A**) 2-*E*-pentenal and (**B**) 2-*E*-hexenal in mayonnaise during storage at 20 °C for up to 4 weeks. For interpretations of code names please refer to **Table 1**.

aroma and flavor were located close to LM0 and LM6. Rancid aroma and flavor descriptors were located between LM12 and LM18 or between LM18 and LM24, respectively. Most variables were found within the inner correlation ring and were therefore not well explained by the model. Hence, the sensory data did not show clear effects of the lactoferrin addition, probably due to the relatively low degree of oxidation observed in the milk drink. PV in weeks 2, 4, 8, and 9 moved toward LM24, indicating that LM24 developed higher PV than the other samples as indicated by the regression coefficients and the raw data. Most of the saturated and monounsaturated aldehydes were located close to LM24 (**Figure 4B**) except in week 9. This was in agreement with the previous finding that aldehydes were present in highest levels in LM24 except in week 9, when the level was often lowest. In contrast, ketones and alcohols were located closer to the other design variables than to LM24.

Mayonnaise. The location of the EDTA design variables to the far right and the location of L16 and L24 to the left in the correlation plot showed that PC1 mainly explained the differences between the EDTA and the lactoferrin samples (**Figure 5**). L32 and L8 and all of the phytic acid design variables were located near the center of the plot, corroborating that these variables were not well explained by this model. The design variable for the sample without metal chelator (NoM) was located in the bottom of the plot. Hence, PC2 explained the differences between the reference and the samples with 16 and

24 μM lactoferrin, which had the most positive PC2 values. PV moved from the fourth quadrant to the left side of the plot (**Figure 5**), signifying that PV developed more slowly in the EDTA mayonnaises compared with the other mayonnaises during storage. Most of the volatile secondary oxidation products were located to the far left with a few exceptions. The finding that L32 and L8 were located close to the center whereas L16 and L24 were located further to the left indicated, in accordance with the analysis of regression coefficients, that volatile formation was most pronounced in L16 and L24. The location of NoM in the bottom of the plot showed that the reference sample developed lower levels of especially 2-pentenal than the lactoferrin and phytic acid samples. Moreover, this location of NoM indicated that the formation of the volatiles located to the left in the plot was at the same level in the reference sample as in the phytic acid and L32 and L8 samples. The location of the EDTA samples to the right clearly showed that the EDTA mayonnaises had lower levels of almost all volatiles compared with the other mayonnaises during the whole storage period.

DISCUSSION

Sensory analysis of the milk drinks did not show clear effects of the concentration of lactoferrin, probably due to the low level of volatiles formed in the milk drink combined with the relatively strong strawberry aroma that was added to make the milk drink more palatable.

In contrast, the PV and volatiles data indicated a concentration-dependent complex effect of lactoferrin on oxidation in both the milk drink and mayonnaise. Thus, high addition levels seemed prooxidative, whereas one of the low addition levels seemed to exert a protective effect.

At the end of the storage period, the PV in the milk drinks had increased in the order LM0 $\approx$ LM6 < LM12 $\approx$ LM18 < LM24, and in mayonnaise the order was E16 $\approx$ E64 $\ll$ L8 < P94 < P16 $\approx$ L16 $\approx$ L24 $\approx$ L32 $\approx$ NoM $\approx$ P126.

The observed increase in PV with increasing lactoferrin concentration could indicate that lactoferrin acted as a prooxidant. However, primary oxidation products are rapidly decomposed in the presence of transition metals (*25*). Thus, this increase in PV with higher concentration of lactoferrin could be a result of increased binding of metals by lactoferrin, which in turn inhibited the decomposition of peroxides. Therefore, to further interpret the effect of lactoferrin, the PV results were compared with data on volatile secondary oxidation products. Most of the volatiles were found in lower levels in the milk drink with 12 μM lactoferrin compared to the levels in LM0 and LM6 samples. Likewise, 8 μM lactoferrin decreased the volatiles formation in mayonnaise. However, in both mayonnaise and milk drink a higher lactoferrin concentration (24 μM) promoted volatiles formation. Taken together, lactoferrin appeared to work optimally as an antioxidant at a concentration of 12 μM in the milk drink and at 8 μM in mayonnaise, whereas it seemed to exert a prooxidative effect at higher concentrations (24 μM).

Lactoferrin is able to bind metals at two sites: (1) a specific binding site, which requires the presence of carbonate ions, and (2) a nonspecific binding site. Addition of bicarbonate ions improved the metal chelator effect of lactoferrin in liposomes and emulsions (*11*). In the absence of synergistic anions, the metal binding sites may repel metal ions (*26, 27*). Thus, a lack of antioxidative effect of lactoferrin in high concentration in milk drink and mayonnaise could result from a lack of synergistic carbonate ions and, thereby, more free metal ions would be in the solution. Additionally, if lactoferrin is located
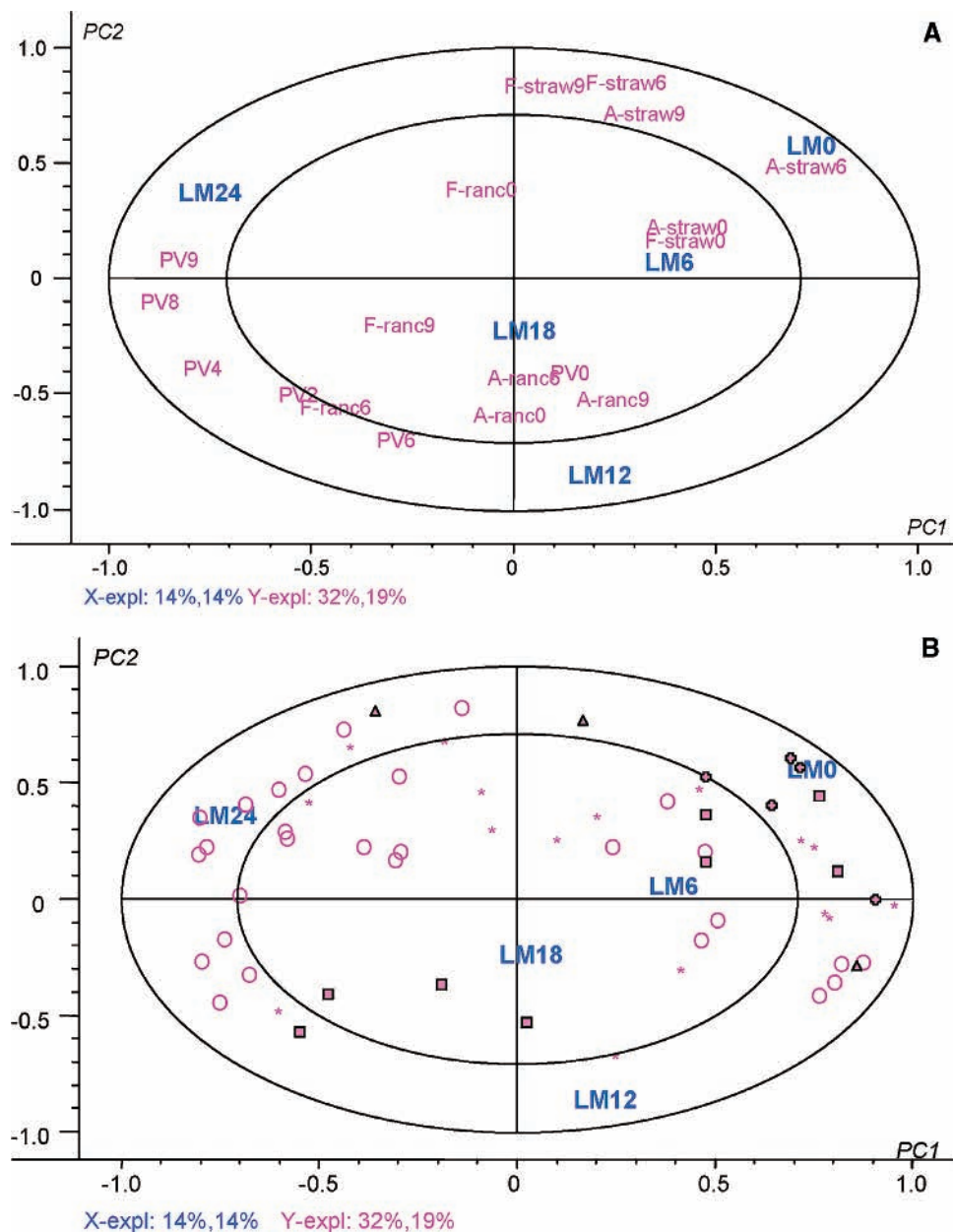
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Figure 4. Correlation loadings plot of PC1 and PC2 from the APLSR analysis on design variables as *X* data and measured variables as *Y* data from milk drink: (**A**) PV and sensory data (F, flavor; A, aroma; straw, strawberry; ranc, rancid); (**B**) volatile secondary oxidation products; (○) aldehyde; (∗) ketone; (□) alcohol; (△) diene; (⊕) pentylfuran. The location of the samples and variables is approximately 0.1 unit on the PC1 axis to the left of the first letter in the sample/variable name. For interpretation of code names and volatiles see **Tables 1** and **4**. The inner ellipse indicates 50% explained variance and the outer ellipse 100% explained variance. The number following the name indicates the week of storage. Replicates are not shown, as they were located in the center of the diagram and therefore confirm that they had no influence. Five principal components were validated. Together these explained 70 and 68% of the variation in te *X* and *Y* data, respectively.

near the oil−water interface and thereby comes in close contact with the lipid droplet in the emulsions, one would expect that the ability of lactoferrin to bind metal ions at sites other than its metal chelating sites could result in prooxidant effects by bringing the metal ions in contact with the lipid (*28*). Moreover, another explanation for the observed prooxidant effect of lactoferrin at high concentrations could be an emulsifying effect of lactoferrin resulting in smaller oil droplets with larger surface area and thus less oxidative stability. Preliminary data indicated that the oil droplets are smaller at higher lactoferrin concentration in milk drink, but further investigations are needed to confirm this.

In a previous experiment with milk drink based on rapeseed oil mixed with specific structured fish oil, no effect of 12 μM

lactoferrin compared to the reference without metal chelator was observed (*15*). Lactoferrin may be denatured during heating (*29*), and we have previously suggested that the inability of lactoferrin to exert an antioxidative effect could be due to partial denaturation of lactoferrin during the production of the milk drink. However, in the present study we did observe a small, but significant, effect of 12 μM lactoferrin in milk drink produced in the same way. Therefore, the lack of a distinct antioxidative effect of lactoferrin in our previous study might be due to the low degree of oxidation in that study.

Previously, we have also demonstrated that 10 μM lactoferrin did not exert any effect on oxidation in mayonnaise based on specific structured sunflower oil (*3*). However, this finding could also be a result of a lower degree of oxidation in this experiment
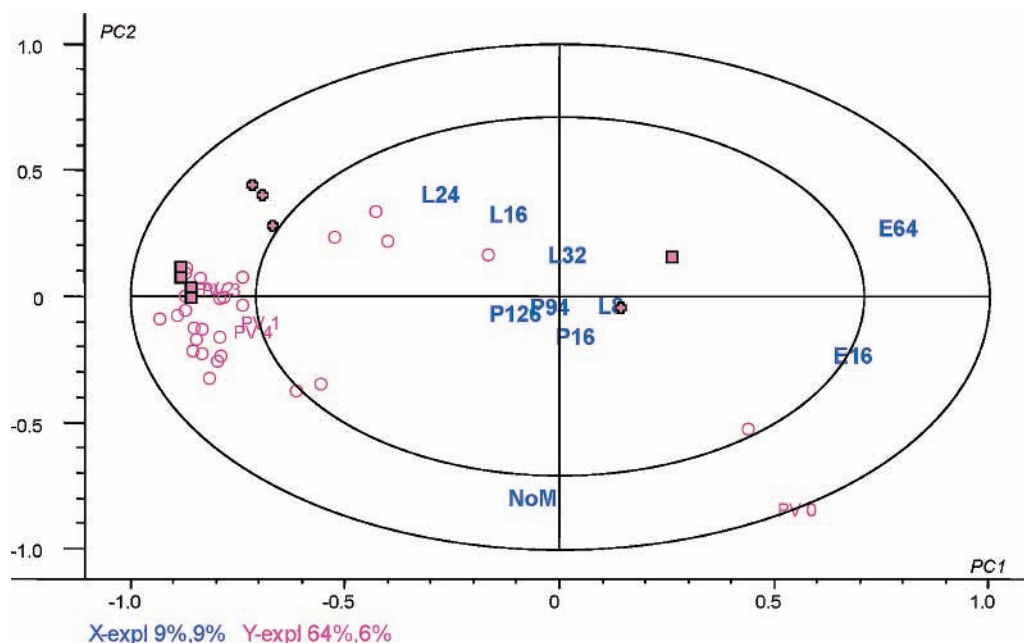
Figure 5. Correlation loadings plot of APLSR analysis on design variables as *X* data and measured variables as *Y* data from mayonnaise: (○) aldehydes; (■) alcohol; (⊕) pentylfuran. The location of the samples and variables is approximately 0.1 unit on the PC1 axis to the left of the first letter in the sample/variable name. Replicates are not shown, as they were located in the center of the diagram, which indicates that they were not explained by PC1 or PC2. Six principal components (PC) were validated in the APLSR model on the data from the mayonnaise storage experiment. Together these six PC explained 54 and 82% of the variance in *X* and *Y*, respectively. Only PC1 and PC2 showed a clear pattern with respect to antioxidant effects, and therefore only these two PC are shown. The design variables in the loadings plot had the same location as the corresponding sample codes in the scores plot, and therefore only the correlation loadings plot is shown.

compared to the present study. In addition, at pH <4, lactoferrin begins to release iron ions and will not bind iron at all at pH ∼2 (*30*). Thus, the low pH of the mayonnaise may have influenced the ability of lactoferrin to bind iron.

To sum up, in both the milk drink and mayonnaise we observed a complex concentration-dependent effect of lactoferrin on the oxidative stability. Interestingly, in the mayonnaise lactoferrin worked as an antioxidant at lower molar chelator/ Fe ratios (1/4:1) compared to the effect in the milk drink (4:1).

Phytic acid added to the mayonnaise did not exert an anti- or prooxidative effect in any of the concentrations employed, even though the metal chelator/Fe ratio tested ranged from $^1/_2$:1 to 4:1. Only a few studies on the effect of phytic acid on oxidation have been reported. In contrast to our results, it has been reported that phytic acid inhibited oxygen consumption in oil/water emulsions (*14*) and that phytic acid inhibited formation of thiobarbituric acid-reactive substances in micelles in ratios of 0.1:1 to 10:1 phytic acid/Fe (*31*). Phytic acid forms a unique iron chelate that becomes catalytically inactive, because the chelate does not retain a reactive coordination site (*32*). Furthermore, phytic acid accelerates oxidation of Fe^{2+}, thereby ensuring a low steady-state concentration of Fe^{2+} (*33*). Thus, an antioxidative effect of phytic acid would be expected. A possible explanation for the lacking antioxidative effect of phytic acid in our study could be a very low formation constant of the Fe−phytic acid complex, but no information about the value of the binding constant is available in the literature. Moreover, the low pH of the mayonnaise may affect the ability of the six phosphoric acid groups in phytic acid to bind the positively charged Fe ions.

Previously, we have demonstrated that the addition of 75 mg/ kg EDTA to fish oil enriched mayonnaise was able to inhibit oxidation (*4*).

Low EDTA concentrations were used in the present study, where EDTA in concentrations as low as 16 μM (corresponding

to an EDTA/Fe ratio of $^1/_2$:1) proved to inhibit the formation of both peroxides and volatiles in fish oil enriched mayonnaise. In simple milk drinks, EDTA in low concentrations (5 mg/kg, ∼0.1 μM) has previously also proved to be efficient in inhibiting oxidation reactions (*5*). Thus, EDTA is a very potent antioxidant in mayonnaise as well as in milk drinks. The efficiency of such low concentrations could be due to a high capacity of EDTA to bind metals. Because EDTA supplies six pairs of electrons it will react with metal ions in a 1:1 ratio (*34*). Prooxidative effects of EDTA have been reported when the ratio between EDTA and iron was less than 1:1, whereas EDTA exerted antioxidative effects when the ratio was higher than 1:1. However, our results show that EDTA is also capable of reducing oxidation when present in ratios lower than 1:1 (EDTA/ metal).

In the present study, the antioxidative effect of EDTA in mayonnaise was much more pronounced than the effect of lactoferrin and especially phytic acid. Thus, lactoferrin was most efficient in a metal chelator/Fe ratio of $^1/_4$:1, whereas the lowest ratio in EDTA mayonnaises ($^1/_2$:1) was even more efficient. Phytic acid in ratios of $^1/_2$:1 to 4:1 did not have any effect on oxidation. As suggested by Huang et al. (*35*) a strong antioxi- dative effect of EDTA may be due to the higher formation constant for the ferric−EDTA complex (1.3×10^{25}) (*10*) compared to the ferric−lactoferrin complex (10^{20}) (*26*, *36*). The availability of a defined lactoferrin preparation and more knowledge of the chemistry and metal-binding activities of these natural compounds are necessary to reveal why lactoferrin exerts a very low antioxidative effect.

In conclusion, the antioxidative effect of EDTA was much more pronounced than the effect of lactoferrin and, especially, phytic acid in mayonnaise. This finding is most likely due to a higher binding constant of Fe^{3+} to EDTA than to the other metal chelators but could also be due to the fact that EDTA seems to be less sensitive to heat and pH values around 4 than the other

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metal chelators. Moreover, whereas lactoferrin seemed to have a slightly antioxidative effect in concentrations of 8−12 µM in milk drink and in mayonnaise, it showed prooxidant activity in higher concentrations.

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